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# A Genetic Approach To Identify *Mycobacterium bovis* Exported Protein Antigens

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# Abstract

A novel approach, combining *phoA*-fusion technology with T cell screening of a recombinant cosmid library, was used to detect *Mycobacterium bovis* exported T cell antigens. An *M. bovis* BCG library of *phoA*-fusions was constructed in *Escherichia coli* and *Mycobacterium smegmatis* using the plasmid vector pJEM11. The *M. bovis BCG* DNA inserts from ten PhoA+ clones were partially sequenced and used to search databases for similarities to known genes. These revealed similarities to a family of genes coding for high temperature-requirement serine proteases and a *Mycobacterium leprae* putative exported lipoprotein gene (*pel*).

The DNA inserts from PhoA+ clones were used to probe an *M. bovis* cosmid library expressed in *M. smegmatis* to identify cosmids containing the full-length genes coding for these exported proteins. Culture filtrates (CFs) prepared from selected *M. smegmatis* recombinants (cosmids) were assayed for their ability to induce proliferation and IFN-γ-production from peripheral blood mononuclear cells (PBMCs) taken from *M. bovis* BCG-immunised and non-immunised control cattle. Culture filtrates from two recombinant *M. smegmatis* (cosmids 44 and 56) induced significant IFN-γ-production and proliferation by PBMCs from immunised animals.

An exported protein gene, identified using the *phoA*-fusion technology, was subcloned from cosmid 56 and its sequence determined and analysed. Database searches using the deduced amino acid sequence of this gene revealed similarities to an *M. leprae* putative exported lipoprotein (Pel) and a family of MalE maltose-binding proteins. The *M. bovis pel* gene was shown to be expressed by recombinant *M. smegmatis*. Preliminary evidence from this study indicates that the *M. bovis* Pel protein is recognised by antigen-specific lymphocytes from *M. bovis* BCG-immunised animals. The PBMCs taken from

*M. bovis* challenged and *M. bovis* BCG vaccinated / challenged cattle also recognised CF from recombinant *M. smegmatis* expressing the *pel* gene in *in vitro* immunoassays.

The combined strategy of using *phoA*-gene fusions and T cell screening of CFs from a recombinant *M. bovis* cosmid library proved a sensitive and rapid method for the detection of potential *M. bovis* T cell antigens.

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## List of Abbreviations

| | |
|---|---|
| 2-D | two dimensional |
| AM | alveolar macrophage |
| APC | antigen presenting cell |
| ATCC | American type culture collection |
| BCA | bicinchoninic acid |
| BCG | bacillus Calmette-Guérin |
| BLAST | basic local alignment search tool |
| bp | base pairs |
| CCT | comparative cervical test |
| CF | culture filtrate |
| CFT | single intradermal caudal fold test |
| c.f.u. | colony forming units |
| CIE | crossed immuno-electrophoresis |
| CMI | cell-mediated immunity |
| cpm | counts per minute |
| dCTP | deoxycytosine triphosphate |
| DEPC | diethylpyrocarbonate |
| DNA | deoxyribose nucleic acid |
| DTH | delayed-type hypersensitivity |
| EDTA | ethylenediamine tetraacetic acid |
| ELISA | enzyme-linked immunosorbant assay |
| G+C | guanine and cytosine |
| hr | hour |
| IFN-$\gamma$ | interferon-gamma |
| kb | kilobases |
| kDa | kilodaltons |
| $Km^R$ | kanamycin resistant |

| | |
|---|---|
| LB | Luria-Bertani |
| MCS | multiple cloning site |
| min | minute |
| MM | minimal medium |
| MOPS | 3-(*N*-morpholino)-propanesulfonic acid |
| MycDB | mycobacterial database |
| OD | optical density |
| ORF | open reading frame |
| PBS | phosphate buffered saline |
| PBS-T | phosphate buffered saline / 0.1% tween-20 |
| PCR | polymerase chain reaction |
| PBMC | peripheral blood mononuclear cells |
| pI | isoelectric point |
| PPD-a | avian purified protein derivative |
| PPD-b | bovine purified protein derivative |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT | room temperature |
| SD | Shine and Dalgarno |
| SDS-PAGE | sodium dodecyl sulphate - polyacrylamide gel electrophoresis |
| sec | second |
| SI | stimulation index |
| SICT | single intradermal cervical test |
| stdev | standard deviation |
| TE | tris-EDTA |
| XP | 5-bromo-4-chloro-3-indolyl phosphate |

# Chapter 1:

# Introduction

## 1.1 Tuberculosis

### 1.1.1 Historical background

No other bacterial genus in history has caused as much human suffering as the genus *Mycobacterium.* The term *Mycobacterium* was first used by Lehmann and Neumann in 1896 to describe the causal agents of tuberculosis and leprosy (Goodfellow and Wayne, 1982). Lesions indicative of spinal tuberculosis have been found in Egyptian mummies dating back to 3700 BC and on a 6000 year old skeleton (Morse *et al*, 1964, cited in Daniel *et al.*, 1994). Skeletal lesions characteristic of leprosy have been found on human remains from AD550 (Grange, 1989).

Tuberculosis, originally known as "phthisis" (derived from the Greek "wasting away") and later known as consumption (Daniel *et al.*, 1994), was first shown to be caused by a transmissible agent by Jean-Antoine Villemin in 1868. However, it wasn't until 1882 that the tubercle bacillus was isolated by Robert Koch (Grange, 1989). The causative organism of leprosy had already been described nine years previously by G. Armauer Hansen but he was unable to culture the leprosy bacillus, which has still not been grown *in vitro* to this day (Grange, 1989; Brooks *et al.*, 1991).

### 1.1.2 The Koch phenomenon

In 1891 Koch published an account of an immunological reaction, now referred to as the "Koch Phenomenon", which distinguished between primary infection and re-infection (Grange, 1989; Brooks *et al.*, 1991). He reported that inoculation of healthy guinea-pigs with virulent *M. tuberculosis* resulted in the formation of a hard nodule after 10-14 days which subsequently turned into an ulcer that rarely healed. In contrast, when *M. tuberculosis*-infected guinea-pigs were inoculated with viable tubercle bacilli, a hard, darkened swelling developed around the site of injection one to two days post-

inoculation. This sloughed off leaving an ulcer which rapidly healed. The same immunological reaction also occurred when the inoculum was in the form of a concentrated, heat-inactivated culture filtrate of the tubercle bacillus; a preparation now known as "Old Tuberculin". Koch's inadvertent discovery of delayed hypersensitivity laid the foundations for the development of the tuberculin skin test by Clemens von Pirquet (von Pirquet 1907 cited in Bothamley and Grange, 1991).

### 1.1.3 Mycobacterial classification

Mycobacteria belong to the family *Mycobacteriaceae* within the order *Actinomycetales*. The G+C content of mycobacterial DNA ranges from 62-70 mol % between species. Morphologically, mycobacteria are slightly curved or straight rods of 0.2-0.6 x 1.0-10 μm in size. Mycobacteria are aerobic, non-motile bacilli that do not form spores (Goodfellow and Wayne, 1982). They possess a thick, lipid-rich envelope (Ratledge and Stanford, 1982), enabling them to resist macrophage killing and chemical injury by acids, bases and antibacterial disinfectants, and giving them the ability to survive long periods in harsh, nutrient-poor conditions without dehydration (Ratledge and Stanford, 1982). The hydrophobic nature of their cell walls also aids in their identification as "acid-fast" bacilli when stained with acid or alcohol (Brooks *et al.*, 1991). The lipid content of mycobacterial cells is high and includes mycolic acids (long chain fatty acids C78-C90), waxes and phosphatides (Brooks *et al.*, 1991).

Mycobacteria are commonly divided into two categories; fast and slow growing species, although no sub-genera are formally recognised. Rapid growing species require less than seven days (typically three to four days) to produce colonies on solid medium from highly diluted inocula (Goodfellow and Wayne, 1982). Slow growers require more than seven days until colonies are visible by the naked eye. Clinically important mycobacterial pathogens fall into the non-cultivable, as in the case of *Mycobacterium leprae*, or the slow growing category, such as the members of the *M. tuberculosis* complex (*M. tuberculosis*,

*Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium africanum* and *Mycobacterium microti*). While this complex is quite separate taxonomically from other mycobacteria, the distinction between its members is less clear prompting the suggestion that they be classified as subspecies of a single species (Goodfellow and Wayne, 1982). Other slow growing mycobacterial pathogens include *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium paratuberculosis (M. avium* subspecies *paratuberculosis)*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, and *Mycobacterium kansasii*. Most of the fast growing mycobacteria, such as *Mycobacterium flavescens* and *Mycobacterium smegmatis*, are non-pathogenic but the opportunistic pathogens of the *Mycobacterium fortuitum* complex (*M. fortuitum* and *Mycobacterium chelonae*) are also rapidly growing species. A phylogenetic relationship between members of the genus *Mycobacterium* was established using 16S ribosomal RNA sequence data (Rogall *et al.*, 1990) and is shown in Figure 1.1 below.

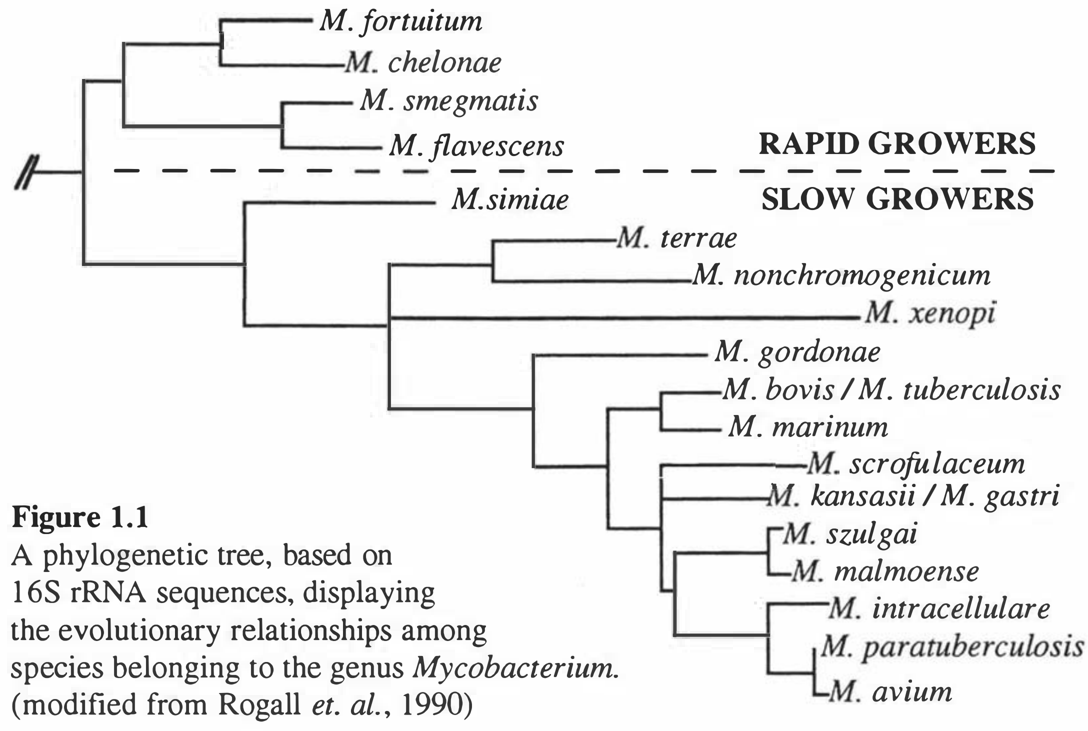



**Figure 1.1**
A phylogenetic tree, based on 16S rRNA sequences, displaying the evolutionary relationships among species belonging to the genus *Mycobacterium*. (modified from Rogall *et. al.*, 1990)

## 1.2 Bovine Tuberculosis

### 1.2.1 History

Tuberculosis or "consumption" has been recognised in cattle since 14AD when the condition was described by Columeela in northern Italy (Wood, 1994). However, it wasn't until Koch's discovery of the tubercle bacillus that the cause of this condition was understood. In 1901 Koch claimed that bovine tuberculosis posed no threat to humans. The controversy created by this unsubstantiated claim led to the establishment of a Royal Commission to investigate the evidence. Based on the data accumulated over the next decade, the Commission concluded that bovine tuberculosis posed a major threat to human health (Grange, 1989). It is now known that the host-range of *M. bovis* is virtually unrestricted in mammals and *M. bovis* can cause disease in humans that is indistinguishable from disease caused by the genetically related *M. tuberculosis*. At present, bovine tuberculosis represents a much lower public health risk than it did prior to the introduction of carcass inspection at meat processing plants, and milk pasteurisation procedures (Livingstone, 1992).

### 1.2.2 Diagnosis and control

The standard method for diagnosing bovine tuberculosis is the tuberculin skin test. Three forms of this test are commonly used; the single intradermal caudal fold test (CFT), the single intradermal cervical test (SICT), and the comparative cervical test (CCT) (Neill *et al.*, 1995). The CFT and SICT are conducted by intradermal injection of *M. bovis* purified protein derivative (PPD) tuberculin into the caudal fold or cervical regions respectively, with the site of injection being examined approximately 72 hours later for signs of swelling and induration. The CCT is similar except that PPD tuberculins from both *M. bovis* and *M. avium* are injected into the cervical region and the skin reactions to each tuberculin are measured and compared. In New Zealand and Australia the CFT is

the standard tool for diagnosis of bovine tuberculosis, with the CCT being used as a supplementary test for animals with borderline results in the initial test (Tweddle and Livingstone, 1994). Animals that have not come into contact with mycobacteria have no immunological reaction to the skin test. In contrast, cattle that have been infected with *M. bovis* develop an indurated swelling. The induration indicates that the host's immune system has previously encountered tuberculin antigens and has developed delayed-type hypersensitivity (DTH; refer section 1.3.2, Acquired immunity). Despite its widespread use, the skin test is not an ideal diagnostic tool due to its inability to distinguish active tuberculosis from past infection or sensitisation by environmental mycobacteria. However, tuberculin skin testing is the universally accepted method for diagnosis of bovine tuberculosis.

The general approach to control of bovine tuberculosis is one of diagnosis and slaughter of tuberculin reactors. Movement control restrictions are then placed on herds containing skin test-positive animals to prevent the spread of disease. Test and slaughter programmes combined with movement control restrictions have been remarkably successful in Australia, Britain, northern Europe and North America in reducing the number of herds on movement control below the 0.2% internationally accepted level (de Lacy, 1995). However, the existence of wildlife reservoirs which contribute to the spread of *M. bovis* infection in cattle has hindered the eradication of the disease in other countries. The badger (*Meles meles*) has been identified as a wildlife reservoir of *M. bovis* infection for cattle in the Republic of Ireland, Northern Ireland and the south-west of England (Neill *et al.*, 1994; Hughes *et al.*, 1996). Red deer (*Cervus elaphus*) are also a potential source of bovine infection in Britain and Ireland. In New Zealand, the Australian brushtailed possum (*Trichosurus vulpecula*) is the major wildlife reservoir, although other feral and wild animals may contribute to the transmission of *M. bovis* to cattle (Ragg and Walker, 1995). Bovine tuberculosis also remains a problem in Brazil and Argentina where pigs are commonly infected with *M. bovis* (Neill *et al.*, 1994).

### 1.2.3 Bovine tuberculosis in New Zealand

Bovine tuberculosis is thought to have been introduced into New Zealand along with cattle in the early-, to mid-nineteenth century (Tweddle and Livingstone, 1994). It soon became established in cattle and by the early twentieth century was considered a serious public health risk. Tuberculin testing of dairy cattle was introduced on a voluntary basis in 1945 and became compulsory for town-supply dairy herds in 1956 (Tweddle and Livingstone, 1994). Within five years this had been extended to all dairy herds and by 1970 tuberculin testing of beef herds was also compulsory (Tweddle and Livingstone, 1994). The test and slaughter program in conjunction with restrictions on the movement of herds containing tuberculin reactors significantly reduced the incidence of disease over most of the country. However, in a few geographically defined areas bovine tuberculosis remained a problem and re-infection was common (Tweddle and Livingstone, 1994). It was clear that cattle to cattle transmission was not the only source of re-infection and in 1971-1972 the possum was identified as a major wildlife reservoir of *M. bovis*.

Possums were introduced into New Zealand for the fur trade in 1858 and were a protected species until 1921 (de Lacy, 1995). Possums are presently considered pests due to the damage they cause to New Zealand's agricultural industry and to the native bush. The possum population has been estimated at anywhere between 50 and 100 million and an eighteen per cent annual kill must be achieved simply to maintain the population at its current level (de Lacy, 1995). When poisoning of possums with sodium monofluoroacetate (1080) became part of the tuberculosis control program the number of infected herds dropped steadily between 1977 and 1981 (Tweddle and Livingstone, 1994). However, the declining incidence of bovine tuberculosis prompted the Government to reduce spending on possum control and the problem re-emerged (Tweddle and Livingstone, 1994).

In 1989 the Animal Health Board was established to oversee the campaign against bovine tuberculosis (Tweddle and Livingstone, 1994). Concerns about overseas consumer perceptions of New Zealand's bovine tuberculosis problem and maintenance of access to export markets became the driving force behind the national tuberculosis eradication program (Tweddle and Livingstone, 1994). By 1991 the extent of the problem prompted a major review of the tuberculosis control scheme. New Zealand was divided into tuberculosis endemic and non-endemic (fringe, surveillance and tuberculosis investigation) areas and new management practices were introduced (Tweddle and Livingstone, 1994). Endemic areas were defined as those where infected wildlife are found or believed to be present on the basis of epidemiological evidence from cattle testing (Livingstone, 1992). Owing to the bush-covered nature of the land and the large number of infected species, it is virtually impossible to eradicate tuberculosis from the six endemic areas using current technology and resources (Tweddle and Livingstone, 1994). While continuation of the test and slaughter programs, movement controls and possum poisoning is vital to halt the spread of disease, research is seen as a pragmatic, economical solution to New Zealand's bovine tuberculosis problem (Livingstone, 1992). One of the research objectives is to develop a cost-effective, internationally acceptable vaccine for possible use in cattle (see Appendix 4). In order to design a successful vaccine, it is first necessary to understand the bovine immune response to *M. bovis* infection.

## 1.3 The Immune Response

The immune response to mycobacteria involves a complex interplay between host, pathogen and the environment, with the outcome being either resistance or disease. Many of the symptoms of tuberculosis are not caused by the tubercle bacilli themselves, but by the immune response of the host to the mycobacteria. Discovering factors that lead to a protective immune response rather than the pathological function is of utmost importance in anti-tuberculosis vaccine design.

### 1.3.1 Innate immunity

Innate, or natural immunity can be defined as the combined functions of physical barriers to infection, cells, and soluble factors in the absence of antigen-specific or acquired immunity (Brown *et al.*, 1994). It is immediate and has no immunological memory. The physical barriers of innate immunity include the skin and mucous membranes. The primary cellular component of innate immunity involves macrophages, neutrophils, and natural killer cells (Brown *et al.*, 1994). Soluble factors include gastric juice, saliva, the components of the complement cascade, and cytokines, such as $\alpha$- and $\beta$-interferons (Goodman, 1991; Rich and Ellner, 1994). The cell surface receptors and soluble protein factors typically recognise carbohydrate structures, such as lipopolysaccharide (LPS) which is a common constituent of Gram negative bacterial outer-membranes.

The first lines of defence against tuberculosis infection are mucous membranes. Transmission of pulmonary tuberculosis is only possible when droplets between 2 and 5 $\mu$m in diameter which contain no more than three viable bacilli are inhaled into the lungs (Dannenberg, 1991). Larger infectious particles settle on the mucosal surfaces of the nasopharynx and the bronchial tree and are moved along by the cilia until they are eventually swallowed (Dannenberg, 1989).

Any tubercle bacilli that pass the physical barriers of innate immunity and lodge on the alveolar membranes are phagocytosed by resident alveolar macrophages (AM's). Whether or not an initial infection develops is due to both the inherent microbicidal properties of the AM and the virulence of the infecting bacilli (Dannenberg, 1989). Phagocytosis may result in bacterial destruction, inhibition or intracellular multiplication. The bacteria are released from the AM if it fails to control their multiplication and neutrophils quickly arrive at the site of infection (Rich and Ellner, 1994). Neutrophils have been shown to be capable of killing *M. tuberculosis in vitro* (Brown *et al.*, 1987) but the precise mechanism by which they do this is unknown. Blood-borne macrophages (monocytes) are recruited to the focus of infection by the chemotactic factors released by neutrophils in response to the mycobacterial infection (Rich and Ellner, 1994). The monocytes then phagocytose the released bacteria but these immature cells are generally unable to restrict the growth of mycobacteria. Natural killer cells may also take part in the early inducible, "non-immune" phase of tuberculosis infection by producing interferon-gamma (IFN-γ) which activates macrophages to express microbicidal functions (Chan and Kaufmann, 1994). During this stage of infection, cell death and tissue necrosis are minimal and the bacilli are able to multiply logarithmically (Dannenberg, 1991). If the innate immune defences fail to rid the host of the mycobacterial pathogen, progress towards disease will occur unless the adaptive, or acquired immune response can restrict bacterial growth. However, the view that innate immunity provides only temporary and incomplete anti-microbial host defence until the development of acquired immunity is too simplistic. The innate and acquired arms of the immune system function in concert and are not separate and unconnected mechanisms of host defence (Brown *et al.*, 1994; Fearon and Locksley, 1996).

### 1.3.2 Acquired immunity

Acquired immunity, also known as adaptive or specific immunity, is mediated by lymphocytes and is characterised by antigen-specificity and memory. There are two facets to acquired immunity; (1) humoral immunity, which is mediated by B lymphocytes, and (2) cell-mediated immunity (CMI), mediated by T lymphocytes.

Humoral immunity

Humoral immunity is mediated by soluble molecules in the blood and tissue fluids of the body, in particular, specific antibodies and complement. Although antibodies against mycobacterial components are produced during infection, they are apparently not protective (Reggiardo and Middlebrook, 1974) and their presence tends to be associated with a disease state. The antibody may opsonize mycobacteria thereby enhancing phagocytosis, although it is not known whether opsonization facilitates mycobacterial elimination.

Cell-mediated immunity

The immune response against tuberculosis infection is highly complex and the antigens and T cell subsets required for protection are not fully defined. The CMI response develops two to three weeks after the initial infection (Dannenberg, 1989). This delay in response is due to the requirement for macrophages, and other antigen-presenting cells (APC's), to display "processed" mycobacterial antigens on their cell surface in association with major histocompatibility (MHC) molecules for recognition by T lymphocytes (Neill *et al.*, 1994; Andersen, 1994a). There are two Classes of MHC molecules involved in antigen presentation; Class I and Class II. Class I MHC molecules are expressed on the surface of all nucleated cells, whereas Class II molecules are found only on the surface of specialised APC's, such as macrophages, B lymphocytes, and dendritic cells. Each Class is involved in the presentation of antigens from a different location. Intracellular antigens are presented to CD8+ T cells in conjunction with Class I

MHC molecules, whereas extracellular antigens are transported through the phagosomes and presented to CD4+ T cells in association with Class II MHC molecules (Germain, 1986). Mature T lymphocytes can be grouped into subpopulations based on the type of cluster of differentiation (CD) antigen expressed on their cell surface (Piessens, 1989). The CD4 molecule is expressed on "helper" T cells while CD8+ T cells generally have a cytotoxic or suppressor function (Piessens, 1989; Young *et al.*, 1990). In this way, the location of an antigen influences the type of immune response that is initiated.

Activation of the antigen-specific CD4+ T helper ($T_H$) cells requires at least two principal signals; the binding of the T cell receptor to the Class II MHC-antigen complex, and the presence of interleukin-1 (IL-1), a lymphokine produced by the APC (Goodman, 1991). Murine studies using long-term T cell clones have shown that CD4+ cells can be subdivided into two classes ($T_{H1}$ and $T_{H2}$) based on their patterns of cytokine production (Mosmann *et al.*, 1986). $T_{H1}$ cells produce IL-2, IFN-γ, and lymphotoxin upon activation, while $T_{H2}$ cells produce IL-4, IL-5, and IL-6, and are associated with the production of antibodies. It is believed that both types of $T_H$ cells are derived from common precursors (Rocken *et al.*, 1992). $T_{H1}$ cells enhance the microbicidal activity of macrophages via the release of IFN-γ. They also elicit the DTH response observed during tuberculin skin testing. DTH is an inflammatory reaction caused by proliferation of, and cytokine release from $T_{H1}$ cells (in response to mycobacterial antigens) which attracts macrophages to the skin test site. Protection of mice against tuberculosis is mediated by $T_{H1}$ cells and IFN-γ is a marker for the $T_{H1}$ response (Huygen *et al.*, 1992; Orme *et al.*, 1992). The division of CD4+ T cells is not as straightforward in humans and cattle, as $T_H$ cells can express an array of cytokines that do not fall into either category. However, $T_{H1}$-like cells are believed to play an important role in protection against tuberculosis, with the generation of a protective immune state involving a balance of $T_{H1}$ and $T_{H2}$ responses.

Activation of the CD8+ T cell also requires at least two signals; the interaction of its T cell receptor with the Class I MHC-antigen complex, and IL-2, produced by the activated $T_{H1}$ cell (Goodman, 1991). Cytotoxic T cells express a similar pattern of cytokine release to that of $T_{H1}$ CD4+ cells (Fong and Mosmann, 1990). These cells are thought to cause the release of mycobacteria from the "protected" environment of the non-microbicidal cell, enabling newly recruited activated macrophages to ingest and destroy the bacilli (Young *et al.*, 1990). Cytotoxic T lymphocytes are believed to provide surveillance for mycobacteria surviving in macrophages and destroy the chronically infected cells by apoptosis (programmed cell death) (Denis *et al.*, 1997).

Another population of lymphocytes involved in the immune response to tuberculosis is the γδ T cell population. The activity of γδ T cells may influence whether a $T_{H1}$ or $T_{H2}$ type of immune response is initiated. The γδ T cell population responds to infection more rapidly than CD4+ or CD8+ T cells, and releases cytokines such as IFN-γ or interleukin-4 (IL-4) depending on the antigenic stimulus (Ferrick *et al.*, 1995). Interesting, γδ T cells are often regarded as part of the innate immune response as they share several features in common with innate immune cells (Boismenu and Havran, 1997). Antigen recognition by γδ T cells resembles the innate immune response in that it is not MHC-restricted, although a few exceptions to this have been noted (Boismenu and Havran, 1997).

There is sufficient evidence to implicate all major T cell populations in the immune response to tuberculosis (Pollock *et al.*, 1996). However, their relative importance and the kinetics of their involvement in the generation of a protective immune response require elucidation. Dynamic changes in the phenotype of the predominant responding T cell population will ultimately determine the host response (Pollock *et al.*, 1996). It is likely that the timing of these changes will depend on several factors, including the nature and dose of the antigen and the genotype and immune competence of the host.

### 1.3.4 The bovine immune response

Much of what is known of the immune response to tuberculosis has come from studies using murine models. This is partly due to a lack of availability of immunological tools for bovines and the difficulty in working with a large, relatively outbred population. However, the increasing body of knowledge on the bovine immune system suggests that it may exhibit similarities to the human immune response to tuberculosis.

There is considerable evidence to demonstrate the central role of T lymphocytes in the bovine immune response against mycobacteria (Fifis *et al.*, 1994). Cattle immunised with *M. bovis* BCG mount a T-lymphocyte proliferative response against a wide spectrum of *M. bovis* BCG culture filtrate proteins (Gulle *et al.*, 1995). Cattle experimentally infected with *M. bovis* also mount a T lymphocyte response against secreted proteins with the immuno-dominance of individual antigens changing over the course of the infection (Fifis *et al.*, 1994).

Protection against virulent *M. bovis* challenge can also be conferred when the *M. bovis* BCG is administered at a low dose (Buddle *et al.*, 1995a; Buddle *et al.*, 1995b). As with mice, the generation of a protective response in cattle is mediated through the activity of antigen-specific T lymphocytes. The immune response can be monitored *in vitro* by T lymphocyte proliferation and γ-IFN production by peripheral blood mononuclear cells (PBMCs) in the presence of *M. bovis* BCG short term culture filtrate proteins and bovine PPD (Carpenter *et al.*, 1995; Gulle *et al.*, 1995). In the murine system, T cell derived IFN-γ has been shown to play an essential role in anti-mycobacterial activity and stimulation of macrophages with IFN-γ reduces growth of *M. bovis* BCG and *M. tuberculosis* (Flesh and Kaufmann, 1987). However, the central role of IFN-γ in the induction of anti-mycobacterial activity in bovine macrophages has yet to be established. A recent study of growth inhibition of *M. bovis* BCG in monocyte-derived macrophages by lymphocytes from *M. bovis* BCG-immunised and non-immunised animals has

indicated that lymphocyte-derived factors, in addition to IFN-γ, are required to activate macrophages to anti-mycobacterial activity (Carpenter *et al.*, 1997). In contrast, lung-derived macrophages treated with IFN-γ alone, can restrict *M. bovis* BCG growth (Aldwell *et al.*, 1997).

Cattle, like humans, appear to possess a high level of natural immunity to tuberculosis. It has been found that γδ T cells make up 20-30% of peripheral blood T lymphocytes in young calves, whereas they are a minority cell type in rodents and humans (Wyatt *et al.*, 1994). The finding that γδ T cells are the first subpopulation of circulating lymphocytes to respond by clonal expansion after *M. bovis* infection provides evidence that γδ T cells play a role in the immune response to bovine tuberculosis (Pollock *et al.*, 1996). The study by Pollock and colleagues suggested that the lymphocyte response to *M. bovis* infection comprises three phases; localisation of γδ T cells to developing lesions and clonal expansion, followed by the sequential involvement of CD4+ and then CD8+ T cells. A recent study investigating the sequential changes occurring at the site of intradermal injection of bovine PPD in tuberculin sensitive cattle also demonstrated the involvement of γδ T cells in the immune response to bovine tuberculosis (Doherty *et al.*, 1996). Two distinct morphological changes were identified: (1) an infiltration of γδ T cells and neutrophils between 6 and 24 hours post-injection, and (2) increased numbers of infiltrating CD4+ cells, CD8+ cells, and macrophages between 24 and 72 hours post-injection (Doherty *et al.*, 1996). However, in spite of their involvement in the immune response to bovine tuberculosis, it is not known if γδ T cells play any role in protective immunity.

## 1.4 The *Mycobacterium bovis* BCG Vaccine

### 1.4.1 *Mycobacterium bovis* BCG

A notable event in the history of tuberculosis research was the development of the Bacille Calmette-Guérin (BCG) vaccine strain. In 1908 Albert Calmette and Camille Guérin, at the Institut Pasteur in Lille, isolated a virulent mycobacterium from a cow with tuberculous mastitis and attenuated the purified strain by serial passage over thirteen years on a glycerol-potato-bile medium (Young and Cole, 1993; Huebner and Comstock, 1994). *M. bovis* BCG was first used as an anti-tuberculosis vaccine for human infants in 1921 (Grange, 1989) and is still used today in many countries and with people of all age groups. It is generally recognised as one of the safest vaccines available although this was seriously questioned after the 1939 tragedy in Lubeck, Germany, when 72 out of 251 *M. bovis* BCG-vaccinated children died of tuberculosis (Bloom and Fine, 1994). However, the *M. bovis* BCG vaccine strain was declared safe when the deaths were shown to be caused by virulent *M. tuberculosis*. The current literature is conflicting as to how this disaster occurred with reports of the vaccine being contaminated with virulent *M. tuberculosis* (Collins, 1988; Bloom and Fine, 1994; Brewer and Colditz, 1995), and claims that a vial of virulent *M. tuberculosis* being used accidentally instead of *M. bovis* BCG (Huebner, 1996). The efficacy of the *M. bovis* BCG vaccine in humans is questionable as various trials have given conflicting results, ranging from 0 to 80% protection (Fine, 1989). This variability has been attributed to differences in prior exposure to environmental mycobacteria, strain variation, genetic and nutritional differences between populations, and climatic conditions (Fine, 1995).

Several physiological differences between virulent *M. bovis* and the attenuated *M. bovis* BCG strains have been noted (Wayne and Kubica, 1986; Roberts *et al.*, 1991). For example, *M. bovis* BCG grows well aerobically and on glycerinated media whereas freshly isolated *M. bovis* grows poorly under these conditions, although *M. bovis* will

adapt to these conditions following repeated subculture (Wayne and Kubica, 1986). Significant differences also exist between various *M. bovis* BCG strains. It has been suggested that *M. bovis* BCG strains be subdivided into two groups on the basis of their secreted protein profiles (Abou-Zeid *et al.*, 1986). The two groups differ in the amount of MPB70 secreted into the culture medium (Harboe and Nagai, 1984), and by the presence or absence of MPB64 (Harboe *et al.*, 1986). Differences between *M. bovis* BCG strains in the mycolic acid patterns obtained by thin-layer chromatography have also been observed (Minnikin *et al.*, 1984). A recent study comparing the immune responses of mice immunised with different *M. bovis* BCG strains demonstrated considerable variation in the immunogenicity of the strains (Lagranderie *et al.*, 1996). However, these differences did not correlate with differences in lipid content or protein profiles.

In addition to the phenotypic and immunogenic differences between *M. bovis* and *M. bovis* BCG, several gross genomic differences have been observed (Mahairas *et al.*, 1996; Philipp *et al.*, 1996). Although the genetic basis for its attenuation is unknown, a recent study comparing virulent *M. bovis* and the vaccine strain *M. bovis* BCG using subtractive hybridisation has revealed three large (approximately 10 kb) regions which are deleted from *M. bovis* BCG (Mahairas *et al.*, 1996). One region was absent from all *M. bovis* BCG sub-strains and present in all virulent *M. bovis* isolates tested suggesting that it may be the original attenuating mutation. However, the role, if any, this region plays in virulence is yet to be established.

### 1.4.2 *Mycobacterium bovis* BCG cattle vaccine trials

Trials to assess the effectiveness of the *M. bovis* BCG vaccine in controlling bovine tuberculosis have been carried out since the development of the vaccine strain (reviewed in Daborn and Grange, 1993). Many of the trials were inconclusive, although most suggest a decrease in the severity of disease in vaccinated cattle (Haring *et al.*, 1930; Waddington and Ellwood, 1972; Daborn and Grange, 1993). The lack of standardisation

of procedures made comparisons between trials difficult and numerous factors, such as vaccine substrain, the dose and route of vaccination and challenge, and prior exposure to environmental mycobacteria, must be considered when evaluating the results of each trial. In particular, interpretation of results from many of the early vaccine trials was complicated by the use of unnatural experimental infection systems. Cattle were challenged with very high doses of *M. bovis* (0.1 to 50 mg; approximately $10^6$ to $5 \times 10^8$ c.f.u.) by the intravenous, subcutaneous and oral routes (Haring *et al.*, 1930; Waddington and Ellwood, 1972). This resulted in the experimentally infected cattle developing generalised tuberculosis with large lesions at multiple sites. The natural disease is rarely this severe and small tuberculous lesions are typically found at only one or two sites (Crews, 1991). Recently, inoculation of cattle by the intratracheal route with a low dose of virulent *M. bovis* (500 c.f.u.) has been found to induce pathology similar to that found in animals with naturally occurring disease (Buddle *et al.*, 1994). This experimental *M. bovis* challenge system has enabled a more realistic assessment of the protective capabilities of the *M. bovis* BCG vaccine.

Antigen dose is known to be critical in determining the type of immune response induced and it has been suggested that low doses of *M. bovis* BCG may be more protective than high doses (Bretscher, 1992). The majority of the cattle vaccination trials used high vaccine doses, ranging from $10^7$ to $10^9$ c.f.u. of live *M. bovis* BCG (Haring *et al.*, 1930; Waddington and Ellwood, 1972; Berggren, 1981). The efficacy of the *M. bovis* BCG (Pasteur) vaccine was recently evaluated using low and medium vaccine doses ($10^4$ and $10^6$ c.f.u., respectively) administered by subcutaneous injection (Buddle *et al.*, 1995a). The cattle were subjected to extensive post-mortem examination five to six months after intratracheal challenge with a low dose (800 c.f.u.) of *M. bovis*. The proportion of animals with tuberculous lesions in the lungs and lymph nodes was significantly lower in the two vaccinated cattle groups than the control group, although there were no significant differences between the low and medium dose vaccine groups. This study clearly demonstrated that *M. bovis* BCG vaccination of calves could induce significant protection

against the development of tuberculous lesions (Buddle *et al.*, 1995a). In a follow-up study, groups of cattle were vaccinated with either *M. bovis* BCG by the subcutaneous or respiratory route or with a killed *Mycobacterium vaccae* preparation by the intradermal route (Buddle *et al.*, 1995b). The animals were subjected to extensive post-mortem approximately five months after intratracheal challenge with 2 x $10^3$ c.f.u. of virulent *M. bovis*, and the degree of protection against the development of lesions was assessed. Killed *M. vaccae* induced no protection against virulent challenge. In contrast, both the numbers of cattle with tuberculous lesions and the number of lesioned lymph nodes in each animal were significantly less for the *M. bovis* BCG vaccinated animals than for the unvaccinated animals. Similar levels of protection resulted from vaccination by the subcutaneous or respiratory routes.

A frequently cited problem with *M. bovis* BCG-vaccination of cattle is that it induces tuberculin reactivity thereby compromising the current diagnostic skin test. Therefore, if *M. bovis* BCG proves effective in controlling bovine tuberculosis, improved diagnostic tools must be developed to distinguish between vaccinated and infected animals.

## 1.5 Mycobacterial Secreted Antigens

### 1.5.1 Evidence for the role of secreted proteins in protection against tuberculosis

The importance of secreted proteins as protective antigens against disease has been established for several pathogenic bacteria. Inoculation with extracellular proteins of the intracellular pathogen *Legionella pneumophilia* has been used to successfully protect guinea pigs against Legionnaire's disease (Blander and Horwitz, 1989). More recently, a recombinant *aro*A$^-$ *Salmonella typhimurium* vaccine expressing two *Listeria monocytogenes* antigens was found to protect mice against listeriosis when the antigens were exported but not when they were expressed as somatic proteins (Hess *et al.*, 1996).

The finding that vaccination with viable *M. tuberculosis*, but not non-living mycobacterial preparations, results in the generation of protective T lymphocytes (Orme, 1988a) is consistent with the hypothesis that secreted proteins are responsible for protective immunity against tuberculosis. It is considered that secreted antigens would be readily available for presentation to T lymphocytes early after infection, whereas the release of somatic antigens depends on prior killing and degradation of the infecting micro-organism. In support of this hypothesis, it has been shown that guinea pigs and mice vaccinated with *Mycobacterium tuberculosis* extracellular proteins show some resistance to virulent challenge (Hubbard *et al.*, 1992; Pal and Horwitz, 1992) and that mice can be efficiently protected against virulent *M. tuberculosis*, by vaccination with a short-term *M. tuberculosis* culture filtrate (CF) using an appropriate adjuvant (Andersen, 1994b).

### 1.5.2 Protein export signals

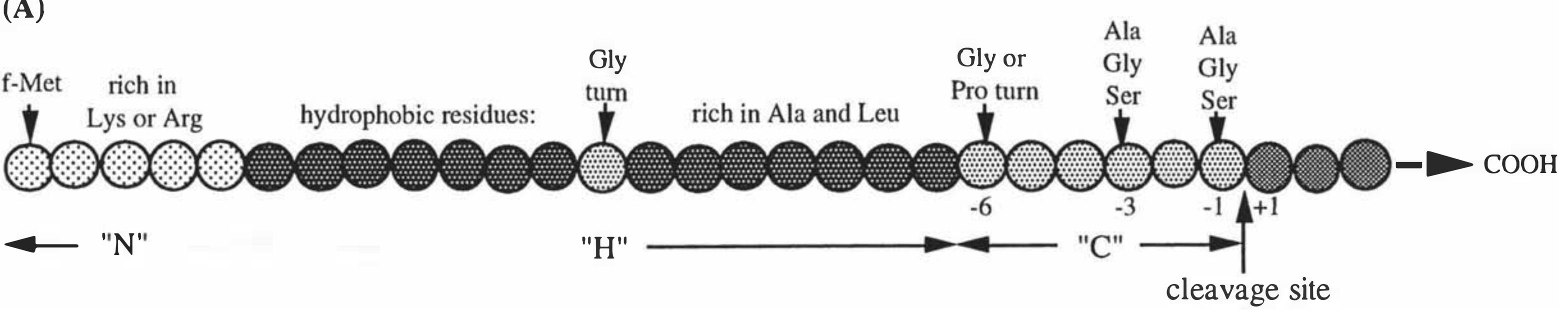
Exported (secreted and cell-associated) proteins transported by the general secretory pathway are synthesised as presecretory proteins with an N-terminal signal peptide that is necessary for translocation across the cytoplasmic membrane. During translocation, the signal peptide is cleaved from the presecretory protein by a membrane-bound signal peptidase. All known bacterial export signal peptides share the same general features. They are typically amino terminal, and have up to three domains, known as the "N", "H" and "C" domains (Pugsley, 1993). The N domain, closest to the N terminus of the presecretory protein, is polar and carries a net positive charge. The N domain of Gram positive signal peptides tends to be longer and more basic than signal peptides from Gram negative organisms (von Heijne, 1992). The N domain is followed by the H domain, or hydrophobic core, consisting of a long alpha-helical stretch (> 8 residues) of hydrophobic amino acids. The H domain frequently ends with an helix-breaking amino acid (proline or glycine). The C domain is less hydrophobic than the H domain and carries the cleavage signals recognised by the signal peptidase (Pugsley, 1993).

Two types of bacterial signal peptidases that cleave the majority of exported proteins have been identified; LepB (SPase I) and LspA (SPase II or lipoprotein signal peptidase) (Pugsley, 1993). A diagrammatic representation of the two types of signal peptides cleaved by these signal peptidases, is shown in Figure 1.2. The majority of signal peptides are cleaved by LepB, which recognises sequences with small amino acids (usually alanine or glycine) at positions -1 and -3 with respect to the cleavage site. A turn-inducing glycine or proline residue is typically found at position -6 with respect to the cleavage site. A glycine-turn is also commonly found within the H domain of standard signal peptides.

Lipoprotein signal peptides (Figure 1.2) are removed following cleavage by LspA. LspA cleaves only those presecretory proteins with a cysteine residue immediately after the

cleavage site. The N-terminal cysteine residue is covalently modified with a glyceride thioether group which anchors the mature lipoprotein to the cell membrane (Hantke and Braun, 1973). Lipoprotein signal peptides tend to be shorter and more hydrophobic than standard signal peptides, and lack the turn-promoting residue commonly found at the end of the H domain (von Heijne, 1989). Lipoproteins have a four residue LspA consensus sequence (L,V,I) (A,S,T,G) (G,A)↓C at positions -3 to +1 with respect to the cleavage site (von Heijne, 1989). Lipoprotein signal peptides require at least one match to this consensus in the first two positions and a precise match in positions -1 and +1. Lipoproteins also have a high incidence of turn-promoting residues (glycine, proline, serine, asparagine, and aspartic acid) downstream of the cleavage site (positions +2 to +6) (von Heijne, 1989). The C domain of lipoprotein signal peptides is apolar and is somewhat shorter than the largely polar C domain of standard signal peptides.

A third bacterial signal peptidase, type IV prepilin peptidase, has been identified (Pugsley, 1993). Unlike LepB and LspA, type IV prepilin peptidase cleaves signal peptides on the cytoplasmic side of the cytoplasmic membrane (Pugsley, 1993). Very few signal peptides are cleaved by this peptidase, which recognises the consensus sequence Q (R,K) G↓(F,M) at positions -3 to +1 with respect to the cleavage site. Cleavage occurs between the N and H domains and thus type IV prepilin signal peptides have no C domain.



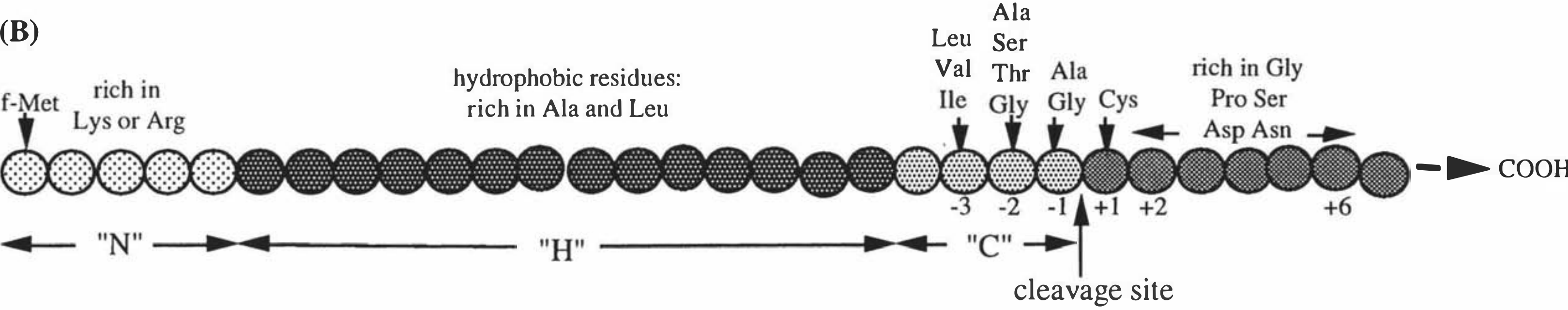




**Figure 1.2**

General features of bacterial signal peptides of presecretory proteins. (A) Standard signal peptide, cleaved by LepB; (B) Lipoprotein signal peptide, cleaved by LspA. "N", "H", and "C" represent the three signal peptide domains. Amino acids are shown using the single letter code. Modified from Pugsley (1993).

### 1.5.3 Mycobacterial culture filtrate antigens

The characterisation of protein antigens is a major focus of tuberculosis research, not only for the identification of antigens as potential vaccine candidates but to identify species-specific or immunodominant antigens for use as diagnostic reagents. Approximately 30 mycobacterial CF antigens have been characterised to date using several different approaches. However, the function of the majority of these proteins is unknown and judging by the large number of proteins observed on 2D gels of mycobacterial culture filtrates (CFs) (Gulle *et al.*, 1995), a great many more antigens remain uncharacterised.

The nomenclature of protein antigens can be confusing, as some antigens are referred to by more than one name. The classification of mycobacterial protein antigens was extensively reviewed by Young *et al.* in 1992 and the properties of mycobacterial proteins were summarised with respect to their immunological significance. This review, which gave each antigen a code number, attempted to eliminate the confusion over nomenclature, and an integrated mycobacterial database, MycDB, has been constructed using the same antigen codes (Bergh and Cole, 1994).

Table 1.1 lists the *M. tuberculosis* and *M. bovis* or *M. bovis* BCG CF antigens of known sequence to date. Several antigens present in mycobacterial culture filtrates do not have signal peptides and are therefore not exported via the general secretory (i.e. signal peptide-dependant) pathway. Such antigens include ESAT-6, superoxide dismutase (SOD), L-alanine dehydrogenase, Des, and members of the heat shock protein family. The number of CF antigens which have been characterised is growing rapidly due to the wide range of approaches now available for identifying secreted protein antigens.

**Table 1.1**

*Mycobacterium tuberculosis*, *M. bovis* and *M. bovis* BCG culture filtrate antigens of published sequence.

| Code Number | Alternative Names | Mol. wt. (kDa) | Proposed Function | Signal Peptide | Selected References |
|---|---|---|---|---|---|
| 1T/B | CIE Ag 63, DnaK | 70 / 71 | Heat Shock Protein | No | [1-3] |
| 2T/B | CIE Ag 82 GroEL, p65, 65 kDa antigen, A60, hsp65, MbaA | 65 | " | No | [1-4] |
| 3T | CIE Ag 78, PhoS, PstS-1, Pab, MPT47, US-Japan Ag 5 | 38 | Phosphate Uptake | Yes | [5-9] |
| 4T | CIE Ag 62, SodA | 23 | Superoxide Dismutase | No | [10,11] |
| 5T/B | GroES, MPB57, BCG-a | 12 | Heat Shock Protein | No | [12-14] |
| 6T | Pac | 44 | L-Ala Dehydrogenase | No | [15] |
| 9T | 35 kDa protein | 35 | - | No | [16,17] |
| 10T/B (a) | CIE Ag 85, Ag85A, MPT44, MPB44, P32, 32 kDa antigen | 32 | Mycolyltransferase carboxylesterase | Yes | [13,18-21] |
| 10T (b) | CIE Ag 85, Ag85B, α-antigen, MPB59, US-Japan Ag6 | 30 | " | Yes | [13,18,19,22] |
| 10T (c) | CIE Ag 85, Ag85C, MPT45 | 31 / 32 | " | Yes | [13,18,19,23] |
| 12T/B | CIE Ag 64, MPT64, MPB64 | 23 | - | Yes | [13,24-27] |
| 13T/B | 19 kDa antigen | 19 | - | Yes | [13,28-32] |
| 15B | CIE Ag 70, MPB70, 22 kDa protein | 18, 22 | - | Yes | [13,33-36] |
| 36T/B | alpha-crystallin, MMP | 14 | Heat Shock Protein | No | [2,37,38] |
| 43T | MPT32 | 41, 45 | - | Yes | [39,40] |
| 46T/B | MPT51, MPB51 | 27 | Fibronectin Binding | Yes | [41] |
| 49T | MPT63 | 18 | - | Yes | [42] |
| - | ESAT-6 | 6 | - | No | [43-47] |
| - | 16 kDa protein | 16 | - | Yes | [48] |
| - | MPT83, MPB83 | 23, 25 | - | Yes | [49-51] |
| - | Erp, exported repetitive protein | 28 | - | Yes | [52-54] |
| - | Des | 37 | Desaturase | No | [52,55] |
| - | PstS-2 | 37, 38 | Phosphate transport | Yes | [9,56] |
| - | PstS-3, CIE Ag 88 | 40 | " | Yes | [8,9,56] |
| - | Apa, 45/47 kDa Ag complex | 45 / 47 | - | Yes | [57,58] |

**Table 1.1 References**

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The protein profile of mycobacterial CF can vary considerably in composition according to the culture conditions (Abou-Zeid *et al.*, 1988; De Bruyn *et al.*, 1989: Andersen *et al.*, 1991). In addition, continued growth beyond mid-log phase often results in contamination of the CF with cytoplasmic proteins, such as Hsp65 and isocitrate dehydrogenase (Abou-Zeid *et al.*, 1988; Andersen *et al.*, 1991). These cytoplasmic proteins can be used as markers to monitor autolysis, and thus give an indication of the quality of the CF preparation. A short-term CF with minimal bacterial lysis comprises proteins that are actively secreted from mycobacteria during the first few days of culture, as well as exported proteins of the cell wall that are gradually released during bacterial growth (Andersen *et al.*, 1991). The gradual release of the exported lipoprotein antigens PstS-1 and MPB83 into the culture medium (Andersen *et al.*, 1991; Harboe *et al.*, 1998), suggests that both cell-associated and purely extracellular forms of these lipoproteins may exist.

Quantitative comparison of protein distribution in mycobacterial CF's and sonicate fractions has been used to study the secretion efficacies of various proteins (Wiker *et al.*, 1991). Wiker and colleagues used a localization index (LI) for each expressed protein, which is defined as follows: LI = concentration (percentage) of total protein in CF / concentration (percentage) of total protein in sonicate. Cytoplasmic proteins have an LI value of near zero, while the LI values of actively exported proteins vary between 5 and 1000. Among those exported proteins studied, the PstS-1 lipoprotein had the lowest LI value (LI = 5) (Wiker *et al.*, 1991). It has been suggested that the variation in LI values of known exported proteins may be due to differences in secretion efficiency or differences in tendency to adhere to the cell surface (Wiker *et al.*, 1991).

**References (this page)**

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### 1.5.4 Strategies for identifying secreted protein antigens

Conventional strategies

Initial strategies aimed at identifying protein antigens were based on biochemical fractionation procedures. In 1949, Seibert made the first attempt at purifying individual mycobacterial antigens by chemical fractionation (reviewed by Daniel and Janicki, 1978). However, the three "purified" proteins described by Seibert were later shown to contain mixtures of proteins (Daniel and Janicki, 1978). Over the next two to three decades, many different fractionation methods were used in an attempt to isolate mycobacterial antigens, but only partial purification was achieved with any single approach (Daniel and Janicki, 1978). Recognising the need to classify these antigens, Janicki and colleagues proposed a reference system for mycobacterial antigens based on immuno-electrophoretic separation of culture filtrate antigens (Janicki *et al.*, 1971). Eleven antigens were identified and named US-Japan Antigens 1-11. This reference system was improved with the technique of crossed immuno-electrophoresis (CIE) using rabbit anti-BCG hyperimmune sera (Closs *et al.*, 1980). Culture filtrate antigens identified using this approach include MPB64, MPB70, PhoS, the 85-complex antigens, and the heat-shock proteins DnaK and GroEL. While these approaches have identified many secreted proteins and laid the foundations for study of mycobacterial culture filtrate antigens, they have two main drawbacks. Firstly, they focused upon B cell antigens, despite the B cell response not being considered of key importance in protection against tuberculosis. Secondly, having identified a B cell antigen, the coding gene remains to be identified. The gene coding for the $\alpha$ antigen (antigen 85-B) has been successfully cloned using an oligonucleotide probe based upon N-terminal amino acid sequence data (Matsuo *et al.*, 1988). This indirect approach for identifying genes coding for secreted antigens has also been used with the CF proteins MPB64, MPB70, and GroES (Terasaka *et al.*, 1989; Yamaguchi *et al.*, 1989a; Yamaguchi *et al.*, 1989b).

Antibody screening of genomic libraries in *Escherichia coli*

A breakthrough in the identification of mycobacterial antigens occurred when the first λgt11 expression library containing recombinant DNA from *M. tuberculosis* was screened for sequences that encoded specific antigens detected by monoclonal antibodies (Young *et al.*, 1985). Using this system, mycobacterial antigens, expressed as fusions with β-galactosidase, are detected by immunoblotting of phage plaques. Other studies have applied this strategy using monoclonal antibodies and sera from hyperimmune animals or human tuberculosis patients, and in a few cases, the antigens have been characterised (Young *et al.*, 1987; Cherayil and Young, 1988; Radford *et al.*, 1988). The advantage of this strategy is that the molecular tools are available for *E. coli* and large numbers of clones can be easily screened. However, this system has several disadvantages:

(1) It relies on the random cloning of DNA fragments downstream of *E. coli* promoters.

(2) It is difficult to distinguish between intracellular and extracellular mycobacterial antigens as proteins are detected in *E. coli* lysates.

(3) Proteins are identified by screening for B cell epitopes.

(4) The *E. coli* clones cannot easily be screened with T cells. Only one laboratory has reported success with screening a λgt11 expression library for T cell antigens (Mustafa *et al.*, 1988). In this study, the screening of 18,000 recombinants yielded a single T cell clone! Other laboratories have reported *E. coli* lysates to contain products which inhibit T cell proliferation (Averill *et al.*, 1993).

(5) If post-translational modifications, such as glycosylation, are required for T cell reactivity, then these T cell antigens will not be detected.

T cell screening of fractionated culture filtrate antigens

A number of strategies aimed at identifying mycobacterial secreted protein antigens have been based upon simple fractionation of CF proteins and testing of protein fractions in immunoassays. The technique of 2-D T cell Western blotting relies on elution of CF proteins following separation by 2-D gel electrophoresis into 96-well tissue culture plates,

and directly testing these samples in lymphocyte proliferation assays (Gulle *et al.*, 1993). Proliferation of bovine T cells in response to fractionated lysate and CF proteins was examined using this strategy and a marked heterogeneity of responses was observed (Gulle *et al.*, 1995). However, none of the antigens identified in this study were purified or characterised. An alternative fractionation strategy, based on elution of proteins from SDS-polyacrylamide gels, also enabled direct testing of fractions in cellular assays (Andersen and Heron, 1993). This approach was successfully used to screen size fractionated *M. tuberculosis* CF proteins to identify two major T cell antigens; the ESAT-6 protein and the previously characterised 85-B antigen (Andersen *et al.*, 1995). Following its identification using this strategy, the ESAT-6 protein was purified and the gene cloned (Sφrensen *et al.*, 1995). These strategies have the advantage that they focus directly on identifying T cell antigens, rather than B cell antigens which must then be evaluated for their T cell reactivity. Dose-response studies are also feasible and enable the optimal concentration of antigens to be established. However, identifying the genes coding for novel T cell antigens requires a return to conventional strategies such as biochemical purification and N-terminal sequencing of the protein, designing oligonucleotide probes based on protein sequence data, or screening expression libraries with antibodies.

Heterologous expression of *M. bovis* and *M. tuberculosis* genes in *M. smegmatis*

Heterologous expression systems using an *M. smegmatis* host have proved extremely useful for the study of mycobacterial secreted protein antigens. They have several advantages over *E. coli* expression systems, such as λgt11 libraries. The major benefit is that most of the gene expression signals of slow-growing mycobacteria are recognised, enabling expression of *M. bovis* and *M. tuberculosis* genes from their natural promoters (Bashyam *et al.*, 1996). In addition, extracellular proteins from *M. bovis* and *M. tuberculosis* are produced by recombinant *M. smegmatis* in a form similar to the native protein and secreted into the culture medium, facilitating purification and direct testing in immunoassays (Laqueyrerie *et al.*, 1995; Roche *et al.*, 1996; Harth *et al.*, 1997). Most

reports using *M. smegmatis* heterologous expression systems have focused on characterising previously identified exported protein antigens. However, two novel strategies based on identifying mycobacterial exported proteins and antigens by using *M. smegmatis* heterologous expression systems have been described (Averill *et al.*, 1993; Lim *et al.*, 1995).

A novel strategy for identifying secreted T cell antigens using an *M. smegmatis* heterologous expression system was described by Averill and colleagues. An *M. bovis* BCG cosmid library expressed in *M. smegmatis* was screened for T cell stimulatory antigens using PBMCs from healthy tuberculin positive donors (Averill *et al.*, 1993). Culture filtrates were prepared from thirty of the *M. smegmatis* recombinants and tested in lymphocyte proliferation assays following analysis by SDS-PAGE. Unique protein bands in three recombinant CFs were observed on SDS-polyacrylamide gels and several CFs induced proliferation of donor lymphocytes. This strategy has also been used to identify bovine T cell antigens from an *M. bovis* cosmid library in *M. smegmatis* (Gormley *et al.*, 1994; Carpenter *et al.*, 1995). There are a number of advantages to this strategy. In addition to the benefits of *M. smegmatis* heterologous expression systems described above, the large insert sizes of individual recombinant cosmids enables the entire *M. bovis* genome to be screened for T cell-reactive antigens in as few as 350 clones. The large insert size also allows gene clusters, or operons, to be expressed (Belisle *et al.*, 1991). When a T cell-stimulatory recombinant CF has been identified using this simple screening system, the gene responsible for the observed immune response can be easily located by subcloning of the cosmid DNA inserts and re-testing CF from the subclones in immunoassays.

A genetic approach for identifying genes coding for exported proteins was recently adapted for use in mycobacteria (Lim *et al.*, 1995). Mycobacterial DNA sequences that direct protein export can be identified by taking advantage of gene fusions to a reporter *phoA* gene of *E. coli*. The PhoA periplasmic enzyme alkaline phosphatase becomes

active when exported across the cytoplasmic membrane to the periplasmic space. Only recombinants that contain DNA inserts with a functional promoter, transcriptional start site and signal sequence (in the correct orientation and reading frame) produce enzymatically active PhoA fusion proteins (Hoffman and Wright, 1985). These clones can readily be detected as they turn blue in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). The *phoA*-fusion technology has been used to identify two novel *M. tuberculosis* exported proteins, Des and Erp (Berthet *et al.*, 1995; Jackson *et al.*, 1997). While this approach has proven highly successful for the identification of novel mycobacterial genes coding for exported proteins, it provides no information on the antigenicity of newly identified proteins. It could, therefore, be more powerful when combined with approaches for identifying T cell antigens.

### 1.5.5 Scope of research

To date, no strategies for screening exported mycobacterial proteins identified using the *phoA*-fusion technology for T cell antigens have been reported. The research presented in this thesis describes a genetic approach for identifying novel *M. bovis* / *M. bovis* BCG exported T cell antigens, based on the *phoA*-fusion technology combined with T cell screening of an *M. bovis* cosmid library. The rationale behind this approach was to exploit the *phoA* reporter gene system to identify novel *M. bovis* BCG sequences directing protein export and map the full-length genes on *M. bovis* cosmids. Culture filtrates from recombinant *M. smegmatis* containing these cosmids can then be screened for their ability to induce an antigen-specific T cell response. Specifically, the ability of CFs from the selected *M. smegmatis* recombinants to induce IFN-γ-production and proliferation by PBMCs from *M. bovis* BCG-immunised and non-immunised control cattle was investigated. It was hoped that this strategy could be used to identify and characterise novel *M. bovis* exported T cell antigens involved in the cell-mediated immune response to tuberculosis.

# Chapter 2:

# Materials and Methods

## 2.1 Bacterial Strains and Plasmids

### 2.1.1 Bacterial strains used in this study

| Bacteria | Source or Description |
|---|---|
| *Escherichia coli* DH10B | Life Technologies Inc., Gaithersburg, MD |
| *M. bovis* BCG strains | ATCC 27290 (Copenhagen), ATCC 35733 (Danish), ATCC 35734 (Pasteur), ATCC 35735 (Montreal), ATCC 35737 (Tokyo), ATCC 35740 (Russia). |
| *M. bovis* KML | New Zealand cattle isolate |
| *M. bovis* 82/8153 | New Zealand isolate * |
| *M. bovis* 83/7561 | New Zealand possum isolate * |
| *M. bovis* ATCC strains | ATCC 19210, ATCC 35724, ATCC 35725 |
| *M. smegmatis* mc$^2$155 | (Snapper *et al.*, 1990) |
| *M. tuberculosis* H37Rv | TMC 102* |
| *M. africanum* | ATCC 25420 |
| *M. avium* | New Zealand veterinary isolate |
| *M. intracellulare* | ATCC 35848 |
| *M. paratuberculosis* | ATCC 53950 |
| *M. kansasii* | ATCC 12478 |
| *M. scrofulaceum* | ATCC 19981 |
| *M. gordonae* | ATCC 14470 |
| *M. marinum* | ATCC 927 |
| *M. terrae* | ATCC 15755 |
| *M. fortuitum* | ATCC 6841 |
| *M. phlei* | ATCC 11758 |

* Kindly provided by G. de Lisle, AgResearch Wallaceville, New Zealand

### 2.1.2 Plasmids used in this study

| Plasmid | Description | Source / Reference |
|---|---|---|
| pJEM11 | *E. coli* - mycobacterial shuttle vector containing a truncated *phoA* gene, $Km^R$ | (Lim *et al.*, 1995) |
| pE2 | pJEM11 containing a 218 bp fragment from BCG | This work |
| pE5 | pJEM11 containing a 510 bp fragment from BCG | " |
| pE8 | pJEM11 containing a BCG DNA fragment of ~1.3 kb | " |
| pE9 | pJEM11 containing a BCG DNA fragment of ~1.0 kb | " |
| pE14 | pJEM11 containing a 347 bp fragment from BCG which overlaps with the insert in pE9 | " |
| pEM1 | pJEM11 containing a BCG DNA fragment of ~1.8 kb | " |
| pEM4 | pJEM11 containing a BCG DNA fragment of ~3.5 kb | " |
| pM2 | pJEM11 containing a BCG DNA fragment of ~3.7 kb | " |
| pM3 | pJEM11 carrying a BCG DNA fragment of ~3.3 kb | " |
| pM4 | pJEM11 carrying a BCG DNA fragment of ~6.3 kb | " |
| pM7 | Identical to pM4 | " |
| pM21 | pJEM11 containing a 1521 bp fragment from BCG | " |
| pYUB18 | *E. coli*-mycobacterial shuttle cosmid vector | (Belisle *et al.*, 1991) |
| pSUM40 | Shuttle *E. coli*-mycobacterial cloning vector containing a *lac* Zα reporter gene, $Km^R$ | (Ainsa *et al.*, 1996) |

## 2.2 DNA Extractions

### 2.2.1 Isolation of plasmid DNA from *E. coli*

Recombinant *E. coli* were grown overnight at 37°C in 5 ml of LB broth containing kanamycin. Plasmids were isolated from *E. coli* by alkaline lysis (Sambrook *et al.*, 1989) or by using the QIAprep-spin Plasmid Miniprep Kit (QIAGEN, Hildon, Germany).

### 2.2.2 Isolation of cosmid DNA from recombinant *M. smegmatis*

*M. smegmatis* recombinant cosmid cultures were grown at 37°C to stationary phase in 7H9 Middlebrook medium supplemented with ADC enrichment (Difco, Detroit, USA) and 20 μg/ml kanamycin (Sigma, St. Louis). Cosmid DNA was extracted based on a method described by Wilson *et al* (1995). Briefly, cultures were incubated for 2 hrs in 100 μg/ml cycloserine, prior to pelleting by centrifugation. The cells were resuspended in 40 mM Tris-acetate / 2 mM EDTA (pH 7.9) and lysed in 20 mM Tris / 1% SDS (pH 12.3). The cell lysates were heated to 30°C for 10 mins, followed by 60°C for 20 mins, then cooled to RT. The DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) in the presence of 50 mM NaCl. Following the addition of 0.5 volumes of 7.5 M ammonium acetate, the sample was centrifuged to remove contaminants. The DNA was precipitated with an equal volume of isopropanol and washed with 70% ethanol. Cosmids were electroporated into *E. coli* and transformants selected on LB / kanamycin plates. The isolated transformants were then grown in LB broth containing kanamycin, and the cosmids extracted using the standard alkaline lysis method (Sambrook *et al.*, 1989). Plasmid DNA was extracted from *M. smegmatis* in the same manner.

### 2.2.3 Isolation of genomic DNA from mycobacteria

The mycobacterial species and strains used in this study are listed in Table 2.1. Mycobacterial cultures were routinely grown in 100 ml of 7H9 Middlebrook medium supplemented with ADC enrichment (Difco, Detroit, USA) with constant shaking at 37°C. Cultures were grown for approximately 3 days or 3 weeks, for rapid- and slow-growing mycobacteria, respectively. Prior to DNA extraction, cultures were heat treated at 70°C for 2 hrs. When cool, streptomycin was added to a final concentration of 100 μg/ml and cultures were left to stand for one hour. Cells were harvested by centrifugation at 5,000 x g for 20 min, washed in pre-lysis solution (25% sucrose, 50 mM Tris pH 8.0, 25 mM EDTA), and incubated at 37°C for 2 hrs in 1.5 ml lysis solution (500 μg/ml lysozyme in pre-lysis solution). A 4.0 ml solution containing 100 mM Tris pH 8.0, 1.0% SDS and 400 μg/ml proteinase K was added and incubation continued at 55°C for 2 hr. After addition of 0.1 ml of 5 M NaCl, the DNA was extracted with phenol-chloroform, and ethanol precipitated at -20°C for 30 min. The DNA was centrifuged at 12,000 rpm for 40 min in a microcentrifuge, washed in 70% ethanol, vacuum dried and resuspended in 0.2 ml of TE buffer (Appendix 1).

## 2.3 DNA Manipulations and Library Construction

### 2.3.1 Manipulations involving DNA

Ligations, restriction digests and electrophoresis of DNA were all carried out as described in Sambrook *et al* (1989) in accordance with the manufacturers' instructions. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim Ltd, Mannheim, Germany.

### 2.3.2 Construction of an *M. bovis* BCG library of *phoA* fusions

*E. coli* DH10B cells were transformed with the pJEM11 plasmid vector. Large scale purification of pJEM11 was carried out by cesium chloride / ethidium bromide equilibrium centrifugation, based on a method described in Current Protocols In Molecular Biology (Heilig *et al.*, 1994). Cultures of *E. coli* (pJEM11) were grown overnight in LB medium (150 ml) containing 20 µg/ml kanamycin. The cells were pelleted by centrifugation at 5,000 rpm for 20 mins at 4°C in a GSA rotor (Sorvall RC5 Centrifuge, Du Pont). Following repeated washing with TE buffer, the cells were resuspended in 12 ml of 25% sucrose / 50 mM Tris / 40 mM EDTA (pH 8.0) supplemented with 1.25 mg/ml lysozyme and 1.5 µg/ml RNAse and incubated on ice for 15 mins. A 7.5 ml volume of 250 mM EDTA (pH 8.0) was added and the cells were incubated on ice for 15 mins. The cells were lysed by addition of 2.5 ml of 5% Triton X-100 / 50 mM Tris / 0.2 M EDTA (pH 8.0) and left on ice for a further 45 mins. Insoluble cellular debris was pelleted by centrifugation (Sorvall SS34 rotor, 15,000 rpm for 90 mins at 4°C). The supernatant was incubated at 65°C for 15 mins and then centrifuged at 7,000 rpm for 15 mins at 4°C, using an SS34 rotor. The supernatants were collected and 1/3 volume of 40% polyethylene glycol / 2 M NaCl added to precipitate the DNA. The DNA was pelleted, resuspended in 15 ml TE buffer, and stored at 4°C overnight. After warming the DNA solution to RT, 12.5 g of CsCl was added slowly while stirring. A

blank solution of 50 g CsCl per 60 ml TE was made to top up the DNA solution to the neck of the ultra-centrifuge tube (Nalgene Ultra Plus, Nalge Nunc International, Rochester, New York) and provide a balance tube. Ethidium bromide was added to both the DNA solution and the blank at a concentration of 0.67 mg/ml. Equilibrium centrifugation was carried out using a Type Ti70 fixed angle in a Beckman L8-70 Ultracentrifuge at 62,000 rpm for 22 hrs 15 mins. The speed of the ultracentrifuge was reduced to 40,000 rpm before stopping after a total of 24 hrs. Under UV illumination, the ultracentrifuge tube was punctured with a 21 gauge needle just below the concentrated plasmid DNA band (below the chromosomal DNA band), and the plasmid DNA collected. The pJEM11 plasmid DNA was washed with an equal volume of TE-saturated isopropanol, and the isopropanol layer, containing the ethidium bromide, was discarded. After four washes, 3 ml TE buffer and 4 ml isopropanol were added to 1 ml of plasmid DNA and the sample frozen at -20°C for 75 mins. The DNA was pelleted at 14,000 rpm for 20 mins at 4°C in a microfuge (Centrifuge 5402, Eppendorf). The DNA pellet was washed twice with 70% ethanol and resuspended in 200 µl TE buffer. Approximately 1 µg of CsCl-purified pJEM11 vector was linearised with the restriction enzyme *Bam HI* and dephosphorylated using *E. coli* alkaline phosphatase (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

Genomic DNA was isolated from *M. bovis* BCG (Pasteur) and partially digested with *Sau* 3AI(~1U per 2.5 µg DNA) for 2.5 mins at 37°C. Following electrophoresis, DNA fragments between 200 and 3000 base pairs were excised from a 1% low melting point agarose gel and purified using GELase (Epicentre Technologies, Madison, Wisconsin) according to the manufacturer's instructions. The *M. bovis* BCG DNA fragments were ligated into the compatible *Bam* HI site of pJEM11 with T4 DNA ligase for 16 hrs at 4°C.

## 2.4 Transformations

### 2.4.1 Transformation of *E. coli*

*E. coli* ElectroMAX DH10B cells (GIBCO BRL, Life Technologies, Inc.) were transformed with purified plasmid DNA by electroporation according to the manufacturer's instructions. The following electroporation conditions were used with the Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA): cells with a 0.2 cm electrode gap, 2.5 kV, 25 μF capacitance, and 200 ohms resistance.

### 2.4.2 Transformation of *M. smegmatis*

*M. smegmatis* mc$^2$155 cells were grown to stationary phase in Middlebrook 7H9/ADC broth containing 0.055% Tween-80 at 37°C in an orbital shaker. Cultures were diluted 1:200 with 200 ml fresh medium and grown to mid-log phase (approximately 16-18 hrs). The cultures were chilled on ice for 30 min then washed twice with ice cold distilled water by centrifugation at approximately 1100 x g for 10 min at 4°C. Cells were washed once with ice cold 10% glycerol and resuspended to a final volume of 1.5 to 2.0 ml. Transformation of 100 μl aliquots of competent *M. smegmatis* cells was carried out by electroporation. The electroporation conditions were the same as described for *E. coli* except transformed *M. smegmatis* were incubated for 3 hrs at 37°C instead of 1 hr prior to plating on selective media.

## 2.5 DNA Sequencing and PCR-Based Amplification

### 2.5.1 PCR cycle sequencing

The DNA inserts from the pJEM11 plasmid constructs were partially or completely sequenced using the Perkin Elmer AmpliCycle™ Sequencing Kit according to the manufacturer's instructions, using [$\alpha$-$^{33}$P]-dCTP label. Labelled DNA fragments were separated on 6% polyacrylamide / urea sequencing gels using BRL Model S2 sequencing gel electrophoresis apparatus (Life Technologies, Inc., Gaithersburg, MD). Plasmid DNA of high purity was prepared from recombinant *E. coli* using the QIAprep-spin Plasmid Miniprep Kit (QIAGEN, Hilden, Germany). DNA primers were designed to the non-coding strand of *phoA* (primer pJ6) and the coding strand of the terminator sequence (primer pJ7) on the pJEM11 vector. Sequencing was carried out in a stepwise manner by designing new primers (Appendix 2B and C) to the newly acquired sequences. Primers were designed and tested for suitability using GeneWorks (release 2.45, IntelliGenetics, Inc. California) PCR Primer Report. Sequencing contigs were built up using the GeneWorks Sequencing Project document.

### 2.5.2 Sequence analysis

DNA and Protein databases were searched using the BLAST algorithm (Altschul *et al.*, 1990) to identify similarities between the insert DNA and previously identified sequences. The BLAST + BEAUTY algorithm (Worley *et al.*, 1995) was also used to search databases for sequence similarities and structural motifs. A DNA database containing sequence data from the Sanger Centre (Tuberculosis BLAST server; http://www.sanger.ac.uk/Projects/ M_tuberculosis/blast_server.shtml) was also searched using *M. bovis* / *M. bovis* BCG insert sequences to identify similarities to the *M. tuberculosis* genome. The Sanger Centre database contains *M. tuberculosis* sequences submitted to the EMBL-database, unsubmitted finished sequences, and unfinished

sequence contigs over a 1000 bases. The GeneWorks programme (release 2.45, IntelliGenetics, Inc. California) was used for all other sequence analyses.

### 2.5.3 PCR amplification of known genes from the *M. bovis* cosmid library

PCR oligonucleotide primers were designed with the aid of the GeneWorks software package based on the DNA sequences of genes coding for known mycobacterial secreted antigens (Appendix 2A). The PCR reactions were carried out using *Taq* DNA polymerase (Boehringer Mannheim) according to the manufacturer's instructions, in the presence of 1.5 mM $MgCl_2$ in a 50 µl volume. The following cycling parameters were used: 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, with an extended elongation step (72°C for 5 min) for the final cycle. Amplification reactions were carried out in a Perkin Elmer 9600 PCR machine.

The PCR amplification products (10 µl samples of each PCR reaction) were analysed by electrophoresis on a 1.2% agarose gel. The molecular size of the amplification products was estimated by comparison with φX174 RF DNA / *Hae* III fragments (GIBCO BRL, Life Technologies).

## 2.6 DNA Hybridisations

### 2.6.1 Isolation and labelling of DNA probes

Plasmid DNA was isolated and purified from recombinant *Escherichia coli* DH10B cells by alkaline lysis (Sambrook *et al.*, 1989). The DNA inserts were purified from the pJEM11 vector by digestion with the restriction enzymes *Kpn* I and *Apa* I. Following electrophoresis, the DNA fragments were eluted from a 1% low melting point agarose gel using a GELase kit (Epicentre Technologies Corporation, Madison, Wisconsin). Restriction fragments were labelled with [$\alpha$-$^{32}$P]-dCTP using the RTS RadPrime DNA labelling system (GIBCO BRL, Life Technologies). This system is based upon the random primer labelling method and produces probes with specific activities of greater than $10^9$ cpm/$\mu$g (Feinberg and Vogelstein, 1983). The nick-translated DNA fragments were separated from unincorporated radiolabelled nucleotides using a NICK column (Pharmacia Biotech, Uppsala, Sweden).

### 2.6.2 Southern blot analysis

DNA restriction digests were size-fractionated on 1.0% agarose gels by electrophoresis and blotted onto nylon membrane (Hybond-N, Amersham International, U.K.). The DNA was fixed to the nylon membrane by U.V. irradiation. Hybridisation was carried out using Rapid-hyb buffer (Amersham) as recommended by the manufacturer. The membrane was incubated at 65$^{o}$C for 4 hr in the rapid hybridisation buffer containing 50-100 $\mu$l of [$\alpha^{32}$P]-labelled probe. Washes were carried out in 2 x SSC (Appendix 1), 0.1% SDS at RT for 10 min, followed by 1 x SSC, 0.1% SDS at 65$^{o}$C for 2 x 15 min. To re-use Southern blots, the membranes were incubated at 45$^{o}$C for 30 min in 0.4 M NaOH and then transferred to a solution containing 0.1 x SSC, 0.1% SDS, 0.2 M Tris-HCl pH 7.5 for 15 min. Autoradiographs were prepared by exposure to X-ray film

(Kodak X-Omat AR) at -75°C for at least 16 hrs in the presence of a single intensifying screen.

### 2.6.3 Colony hybridisation

Colony hybridisation was carried out using nylon membrane discs (Hybond-N+ Nucleic Acid Transfer Membrane discs, 0.45 µm removal rating; Amersham, Little Chalfont, England). Recombinant *M. smegmatis* (cosmids) colonies were patched onto nylon membrane discs placed on top of 7H10 agar plates containing Middlebrook OADC enrichment (Difco, Detroit, USA) and 30 µg/ml kanamycin. Following overnight incubation at 37°C, the *M. smegmatis* recombinants were lysed by floating discs on a solution containing 0.5 M NaOH and 1.5 M NaCl. Membrane discs were neutralised in 1 M Tris / 1.5 M NaCl / pH 7.5 and the DNA was fixed by placing discs in a microwave oven for 1 min.

## 2.7 Protein Preparations

### 2.7.1 Preparation of culture filtrates

The *M. bovis* cosmid library was constructed as described previously (Wilson and Collins, 1996) and kindly provided by Dr. D.M. Collins. High molecular weight *M. bovis* genomic DNA was partially digested with *Sau* 3AIand the fragments separated by centrifugation on a 10-40% sucrose density gradient. Fragments of 30-40 kb were ligated into the compatible *Bam* HI cloning site of the pYUB18 vector, packaged into phage particles and electroporated into *E. coli.* The cosmid library was extracted from the pooled kanamycin-resistant transformants and electroporated into *M. smegmatis.* Approximately 350 *M. smegmatis* recombinants were patched onto 7H10 agar plates containing Middlebrook OADC enrichment (Difco, Detroit, USA) and 30 µg/ml kanamycin and stored as glycerol stocks. Cosmid DNA was extracted from all 350 clones and used to transform *E. coli* by electroporation to facilitate analysis.

Recombinant *M. smegmatis* were cultured in a modified version of Middlebrook 7H9 (Difco, Detroit). The broth was made up as a minimal medium (MM) by leaving out the ADC enrichment and tween-80. Following autoclaving, the medium was supplemented with 0.2% dextrose. Cultures were initially grown to stationary phase in 5 ml of MM + dextrose containing 20 µg/ml kanamycin at 37°C and then inoculated 1:40 into fresh medium and grown for 5 days. The cells were removed by centrifugation at 5000 x g for 10 min and the culture supernatant filtered through a 0.2 µm membrane (Millipore, Bedford). The culture filtrate (CF) was then concentrated on a 'centriplus' filter with a 3 kDa cut-off (Amicon Division, Beverly, USA) to a final volume of approximately 1.5 ml. Protein concentrations were determined using a BCA colourimetric micro assay kit (Pierce, Rockford).

Culture filtrate of *M. bovis* BCG (Pasteur) was prepared based on the method described by Gulle *et al* (1995). Briefly, *M. bovis* BCG was grown in modified Sauton's medium on an orbital shaker for 14 days. Following removal of bacteria by centrifugation and filtering of the supernatant through a 0.2 μm membrane (Sartolab P20, Sartorius AG, Goettingen), the CF was concentrated in a stirred ultrafiltration cell with a 10 kDa cut-off filter (YM10: Amicon Division, Beverly, USA) to a final volume of approximately 2.5 ml. The concentrated CF was washed three times with double-distilled water, and Tris-HCl (pH 8.8) and $MgCl_2$ were added to final concentrations of 20 mM and 2 mM, respectively. The CF was then treated with 2 μg/ml DNAse I and 1 μg/ml RNAse for 10 mins at RT.

To prepare CF samples of recombinant *M. smegmatis* clones (pM21 and pJEM11) from 1 L cultures, recombinants were initially grown in 10 ml of Middlebrook 7H9-ADC (Difco, Detroit) containing 20 μg/ml kanamycin at 37°C on an orbital shaker for 4 days. Cultures were washed three times with 7H9-MM supplemented with 0.2% dextrose and kanamycin at 37°C by centrifugation at 3000 x g for 10 min at RT. The washed recombinant *M. smegmatis* were inoculated into 1 L of fresh 7H9-MM medium containing dextrose and kanamycin and grown incubated with constant shaking at 37°C for 7 days. Cells were pelleted by centrifugation at 5000 x g for 10 min and the culture supernatant filtered through a 0.2 μm membrane (Sartolab P20, Sartorius AG, Goettingen). The CF was concentrated using a stirred ultrafiltration cell with a 10 kDa cut-off filter to a final volume of approximately 2.5 ml.

### 2.7.2 Preparation of cell lysates

*E. coli* lysates

Recombinant *E. coli* clones were grown in LB culture medium supplemented with 20 μg/ml kanamycin at 37°C on an orbital shaker for approximately 7 hrs. Cultures were inoculated 1:1000 into 25 ml of fresh medium and grown overnight. The cells were

washed twice with 25 mM Tris-HCl / 2 mM $MgCl_2$ (pH 7.0) buffer by centrifugation at 3000 x g for 10 min at 4°C, and resuspended in 5.0 ml of wash buffer. Cells were sonicated on ice for 4 x 1 min at 30 sec intervals using an XL-2020 Sonicator (Heat Systems Inc., Farmingdale, NY) on setting 3.5. Insoluble debris was pelleted at 14,000 rpm for 10 min at 4°C in a microfuge and the supernatants collected. When necessary, proteins were concentrated by precipitation with 8 volumes of acetone at -20°C overnight (Powell, 1994). A non-recombinant *E. coli* DH10B lysate control sample was prepared according to the above protocol from a 25 ml culture in the absence of kanamycin.

*M. smegmatis* lysates

Lysate samples of recombinant *M. smegmatis* clones (pM21 and pJEM11) prepared from 1 L cultures were grown as described in section 2.7.1. Cell pellets were resuspended in 20 ml fresh 7H9-MM medium containing 100 μg/ml cycloserine and incubated at 37°C overnight. The cells were washed twice in phosphate buffered saline (PBS; pH 7.4) by centrifugation at 3000 x g for 10 min at 4°C, and resuspended in 5.0 ml of PBS containing 10 μg/ml lysozyme. Cells were sonicated on ice for 5 x 1 min at 30 sec intervals using an XL-2020 Sonicator with microtip on setting 3.5. Approximately 200 units of DNase I (BRL, Life Technologies, Auckland) and 40 μl of 500 mM EDTA (pH 8.5) were added to the cell lysates. Insoluble cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C in a microfuge.

Recombinant *M. smegmatis* lysate samples were also prepared from cells scraped from solid media. The *M. smegmatis* recombinants were streaked from glycerol stocks onto LB / kanamycin plates and grown at 37°C for three to four days. Colonies were scraped from plates in a 1.5 ml volume of PBS (pH 7.4) using a glass rod. The cells were pelleted in a microfuge and the supernatants (extra-cellular wash samples) removed. The *M. smegmatis* cells were washed twice with PBS (pH 7.4) and resuspended in 1 ml of PBS. Crude cell lysates were prepared by sonication on ice for 2 x 15 secs at 15 sec intervals using an XL-2020 Sonicator with microtip on setting 3.5. Samples (20 μl)

were boiled in SDS-polyacrylamide gel loading buffer (Appendix 1), pelleted in a microfuge, and the supernatants analysed on SDS-polyacrylamide gels.

## 2.8 Protein Analysis

### 2.8.1 Estimation of protein concentration

The protein concentrations of CFs from recombinant *M. smegmatis* and *M. bovis* BCG were estimated in duplicate using the BCA Protein Estimation Assay (Pierce, Rockford) according to the manufacturer's instructions. The BCA assay is based upon a colour change resulting from a complex formed between $Cu^{+}$ and bicinchoninic acid (Smith *et al.*, 1985). The $Cu^{+}$ is produced by oxidation of cysteine, tyrosine, and tryptophan residues, and by a temperature-dependent reaction of peptide bonds with $Cu^{2+}$.

### 2.8.2 Alkaline phosphatase assays

Crude extracts of recombinant *M. smegmatis* were prepared from freshly streaked colonies scraped from LB / kanamycin plates in a small volume of phosphate buffered saline (PBS). Cells were pelleted by centrifugation and the supernatants removed for assay. Cells were washed twice with PBS then resuspended in 5 ml 1M Tris-HCl (pH 8.0) and disrupted for 2 x 15 secs at 15 second intervals using a sonicator with microtip on 3.5 setting. Alkaline phosphatase assays were carried out on sonicated extracts based on a method previously described by Kremer *et al* (1995). Sonicated extracts (100 µl aliquots) were assayed in 900 µl of 1M Tris-HCl (pH 8.0) and 100 µl of 20 mM *p*-nitrophenylphosphate (Sigma, St. Louis). Enzyme reactions were carried out in triplicate at 37°C in the dark. Assay reactions were stopped by the addition of 100 µl of 1 M $K_2HPO_4$, and the optical density was measured at 420 nm. A standard curve was constructed over a range of $5.0 \times 10^{-5}$ to $1.7 \times 10^{-3}$ PhoA units using *E. coli* alkaline phosphatase (Pharmacia Biotech, Uppsala, Sweden). The protein concentration of each sample was measured to allow for direct comparison between samples.

Alkaline phosphatase activity was also detected, but not quantified, in recombinant *E. coli* and *M. smegmatis* colonies grown on solid media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP) (Sigma, St. Louis).

### 2.8.3 Protein analysis by SDS-PAGE and Western blotting

Protein separation was carried out on 8.0% or 10.0% polyacrylamide gels (SDS-PAGE) with a 5% stacking gel of pH 6.8. The electrophoresis buffer consisted of 192 mM glycine, 25 mM Tris and 0.03% SDS (Laemmli, 1970). Broad range molecular weight proteins were used as size standards (Bio-Rad Laboratories, Auckland). Proteins were visualised by staining with Coomassie Brilliant Blue R-250, followed by silver-staining.

For Western blotting experiments to detect PhoA fusion proteins, protein samples were run on an SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (0.2 µm pore size; Micro Filtration Systems, USA) using a mini-Transblot apparatus (Bio-Rad Laboratories, Auckland) at 20 V for 60 mins. Approximately 60 µg and 120 µg of lysate samples from recombinant *E. coli* and *M. smegmatis*, respectively, and 150 µg of the recombinant *M. smegmatis* CF samples were loaded on the gels. Rainbow molecular weight standards (Amersham) were run on gels alongside protein samples. The membranes were incubated in a 2% milk powder solution in PBS / 0.1% Tween-20 (PBS-T) for 2 hrs at RT to block sites without bound protein. Membranes were incubated with rabbit anti-*E. coli* alkaline phosphatase IgG (# 200-4134, Rockland, Gilbertsville, USA) diluted 1:5,000 at 4°C overnight. Following washing with PBS-T, membranes were incubated with goat anti-mouse IgG-alkaline phosphatase conjugate (# 100H-8826; Sigma, St Louis) diluted 1:2,500 at RT for 1 hr. The membranes were washed twice with PBS-T and once with substrate buffer (100 mM Tris / 100 mM NaCl / 10 mM $MgCl_2$). Protein bands detected by the antibody were visualised by the addition of 50 µg/ml XP and 0.01% nitro-blue tetrazolium as substrates (Sigma, St. Louis).

For the Western blot experiment used to detect MPB64 in CF samples from *M. bovis* and recombinant *M. smegmatis*, rabbit anti-MPB64 polyclonal sera (kindly supplied by P.R. Wood, CSIRO, Parkville, Australia) was used at a 1:500 dilution, and goat anti-rabbit IgG alkaline phosphatase conjugate (# A1418; Sigma, St Louis) was used at a 1:2,500 dilution.

## 2.9 Immunoassays

### 2.9.1 Immunisation of cattle with *M. bovis* BCG

All cattle used in this study were selected on the basis of low responses to *M. smegmatis* CF and avian purified protein derivative (PPD-a), as measured by lymphocyte proliferation. Four male yearling Freisians (animals 5, 12, 15 and 21) were injected subcutaneously on two occasions, 10 weeks apart, with approximately $10^5$ c.f.u. of *M. bovis* BCG (Pasteur) in PBS. Animals 5 and 12 were given a third immunisation 26 weeks after the second immunisation, as their immune responses to *M. bovis* BCG CF had diminished. Control cattle were age and sex-matched non-immunised animals.

### 2.9.2 Lymphocyte proliferation assays

Peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood samples by density centrifugation over lymphoprep (density = 1.077 g/ml; Nycomed Pharma As., Oslo, Norway). The PBMCs were washed by centrifugation three times in PBS and resuspended at a concentration of $2 \times 10^6$ / ml in supplemented RPMI-1640 culture medium (Gibco BRL, Auckland, New Zealand) (Gulle *et al.*, 1995). The PBMCs were cultured in quadruplicate wells of 96-well flat-bottomed microtitre plates (Nunc, Roskilde) in the presence of recombinant *M. smegmatis* culture filtrate proteins, in a total volume of 200 µl. The cells were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ for 5 days. Proliferation was detected by pulsing each well with 0.5 µCi of [$^3$H] thymidine (Amersham, Sydney, Australia) for the final 16 hrs. Cells were harvested on to glass fibre filter-mats (Pharmacia, Turku, Finland) and the incorporated radioactivity measured in an LKB 1205 Betaplate liquid scintillation counter. Results were expressed as the mean counts per minute (cpm), or as stimulation indices (SI), which are the mean cpm of replicate stimulated wells divided by the cpm of non-

stimulated wells. Values outside the median value of replicate wells +/- 50% were disregarded when calculating the mean cpm and SI as recommended (Stone *et al.*, 1991).

### 2.9.3 IFN-γ assays

Peripheral blood mononuclear cells were prepared and incubated with CF samples from recombinant *M. smegmatis* as described for the lymphocyte proliferation assay. Supernatant (50 μl) was harvested from each of the replicate culture wells on day 5 of incubation, pooled, and frozen at -20°C until required. The presence of IFN-γ in the supernatants was determined in duplicate using a sandwich enzyme immunoassay for bovine IFN-γ (Commonwealth Serum Laboratories, Parkville, Australia) (Rothel *et al.*, 1990).

The IFN-γ concentration of the recombinant positive control IFN-γ sample supplied with the ELISA kit was determined using a standard curve of recombinant bovine IFN-γ (kindly supplied by Ciba-Geigy, Basel, Switzerland). The optical densities of sample wells were measured at 450 nm, and the IFN-γ concentrations (units/ml) calculated using a standard curve of the IFN-γ positive control sample supplied with the kit.

## 2.10 Gene Expression Studies

### 2.10.1 Isolation of total RNA from recombinant *M. smegmatis*

Recombinant *M. smegmatis* (subclones 56/1 and 56/2) cultures were grown at 37°C to late log phase (OD 670 nm = 0.9) in LB medium supplemented with 0.5% glycerol, 0.2% Tween 80 and 20 μg/ml kanamycin. Total RNA was extracted from 3 ml cultures using an RNA isolation kit (High Pure, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

### 2.10.2 Northern blot analysis

The RNA was electrophoresed and transferred to a nylon membrane as described in Rueger *et al,* 1996. Approximately 15 μg of each RNA preparation was run on a 1% agarose-MOPS formaldehyde gel (Appendix 1) using 1 x MOPS running buffer (Appendix 1) containing 1 mg/ml ethidium bromide. An RNA size standard (GIBCO BRL, Life Technologies) was run alongside the mycobacterial RNA samples. Following electrophoresis, the gel was equilibrated in 20 x SSC and transferred to a nylon membrane. The RNA was fixed to the membrane by UV illumination. A 234 bp fragment of the *pel* gene was generated by digestion of pM21 with *Kpn* I and *Pvu* II and labelled as described in section 2.6.1. Hybridisation was carried out for 16 hrs at 65°C using 150 μl of [$\alpha^{32}$P]-labelled probe in approximately 7 ml of pre-warmed Rapid-hyb buffer (Amersham International, U.K.). Two, 30 min washes at 65°C were carried out in each of the following solutions: 1 x SSC / 0.1% SDS, followed by 0.7 x SSC / 0.1% SDS, and 0.1 x SSC / 0.1% SDS. Autoradiographs were prepared by exposure to X-ray film (Kodak BioMax MR) at -75°C for 7 hrs in the presence of a single intensifying screen.

# Chapter 3:

# Identification And Analysis Of DNA Sequences Coding For Protein Export Signals

## 3.1 Abstract

An *M. bovis* BCG library of *phoA*-fusions was constructed using the *E. coli* / mycobacterial shuttle vector pJEM11. Recombinant *E. coli* and *M. smegmatis* expressing enzymatically active PhoA fusions were identified by plating transformed cells onto media containing the substrate XP. *M. bovis* BCG DNA inserts from selected blue colonies were partially sequenced. One sequence contained an ORF identical to an *M. leprae* ORF. A second ORF showed similarity to a family of high temperature requirement serine proteases.

The PhoA fusion proteins encoded by the *M. bovis* BCG/pJEM11 plasmid constructs were detected in *E. coli* lysate samples by probing Western blots with anti-PhoA antibody. The antibodies detected proteins in all samples from PhoA+ recombinants. These protein bands were of higher molecular weight than those detected in the non-recombinant *E. coli* lysate sample and the PhoA positive control.

PhoA fusion proteins were also examined in recombinant *M. smegmatis*. The antibody detected proteins in each PhoA+ lysate sample. Culture filtrate and lysate samples prepared from 1 L cultures of selected recombinant *M. smegmatis* were also analysed by Western blotting for the presence of PhoA fusions. The antibody detected two protein bands of the similar molecular weight in a recombinant *M. smegmatis* CF and lysate sample. These bands were absent from control samples.

## 3.2 Introduction

The secreted antigens of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG are of particular interest in view of their capacity to induce a protective immune response (Orme, 1988a; Andersen *et al.*, 1991). Exported proteins which remain attached to the cell membrane may also play an important role in infection, during the interactions of the mycobacterium with the host cell. In spite of their importance, relatively few exported (secreted and cell membrane-associated) proteins have been characterised. This is primarily due to the difficulties associated with screening large numbers of proteins and relating immune responses to individual antigens and their coding genes. An alternative strategy is to firstly identify genes which code for exported proteins and determine whether the gene products possess T cell epitopes. Genes encoding such exported proteins can be identified by creating a library of fusions to a reporter *phoA* gene, coding for the *E. coli* periplasmic enzyme alkaline phosphatase. The enzyme is synthesised as a precursor monomer which contains a signal peptide at the N-terminus. The signal peptide is removed during export to the periplasmic space and two mature monomers dimerize to form the active enzyme. As PhoA retained in the cytoplasm is enzymatically inactive (Derman *et al.*, 1993), only PhoA fusions which contain signal peptides will be detected in alkaline phosphatase assays.

An *E. coli*-mycobacteria shuttle plasmid, pJEM11 (Figure 3.1), has been constructed for the purpose of screening mycobacteria-*pho*A gene fusion libraries for sequences that direct export of alkaline phosphatase (Lim *et al.*, 1995). The plasmid contains both *E. coli* and mycobacterial origins of replication, a truncated *pho*A gene (devoid of a promoter, start codon and signal sequence) downstream of a cloning site, and a kanamycin resistance gene as a selectable marker. This vector has been used to construct a library of *M. tuberculosis phoA* fusions (Lim *et al.*, 1995). Twelve inserts coding for sequences directing protein export were identified. The open reading frames (ORFs) included those coding for the exported 19-kDa lipoprotein, a homologue of the 28-kDa

protein of *M. leprae*, and a protein with similarity to stearoyl-acyl-carrier-protein desaturases (Lim *et al.*, 1995).

This chapter describes the construction of an *M. bovis* BCG library of phoA-fusions using the vector pJEM11, to identify novel *M. bovis* BCG exported protein genes. *M. bovis* BCG DNA inserts directing the export of PhoA protein fusions were partially sequenced and the deduced amino acid sequences of selected ORFs were examined to identify export signals.

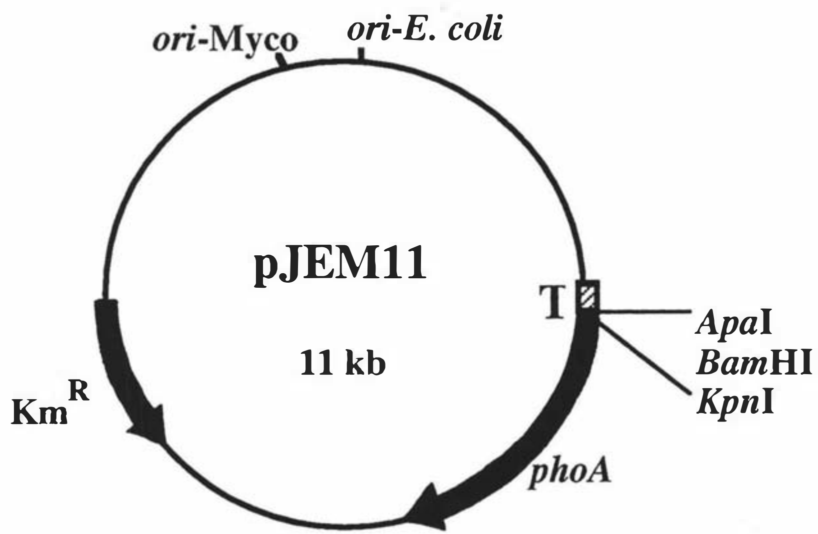



**Figure 3.1**

The *E. coli*-mycobacterial shuttle plasmid, pJEM11. The plasmid was constructed in Dr. Brigitte Gicquel's laboratory (Unité de Génétique Mycobactérienne) at the Institut Pasteur, Paris. $Km^R$, kanamycin resistance gene; *phoA*, truncated *phoA* gene (encoding the enzyme alkaline phosphatase) devoid of a promoter, start codon and signal peptide; T, transcriptional terminator; *ori*-Myco and *ori-E. coli*; origins of replication for mycobacteria and *E. coli* respectively.

## 3.3 Results

### 3.3.1 Construction and screening of an *M. bovis* BCG library of PhoA-fusions

An *M. bovis* BCG library of *phoA*-fusions was constructed using the vector pJEM11 and expressed in *E. coli* and *M. smegmatis* as described in Materials and Methods (Chapter 2.3.2). A flowchart outlining the library construction and screening process is shown in Figure 3.2. Plasmid DNA was purified from twelve randomly selected *E. coli* transformants and digested with *Pst* I and *Kpn* I to estimate the percentage of the library that contained DNA inserts. Eleven of the twelve plasmids were found to contain DNA inserts, suggesting that approximately 92% of the library was recombinant (data not shown). The DNA insert sizes ranged from approximately 230 to 2000 base pairs (bp) with an average insert size of 500 bp. The majority of these DNA inserts were small; only one recombinant contained an insert of greater than 500 bp in size.

The pJEM11/*M. bovis* BCG genomic library was screened for recombinants encoding exported PhoA fusion proteins by plating *E. coli* transformants onto medium containing kanamycin and the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). Recombinant clones producing enzymatically active PhoA fusion proteins turned blue in the presence of this substrate. Approximately 8000 transformants were obtained after plating 7.5% of the library. Fourteen turned blue within 24 hours (plasmids designated pE1 to pE14) under conditions where the negative control (*E. coli* pJEM11) remained white. Seven of the fourteen recombinant *E. coli* remained positive for PhoA activity upon restreaking for single colonies (Figure 3.3). Restriction endonuclease analysis of plasmid DNA prepared from these *E. coli* recombinants revealed that their inserts ranged from approximately 0.3 to 3.3 kilobases (kb) in length (Figure 3.4) with a mean size of 1.2 kb.

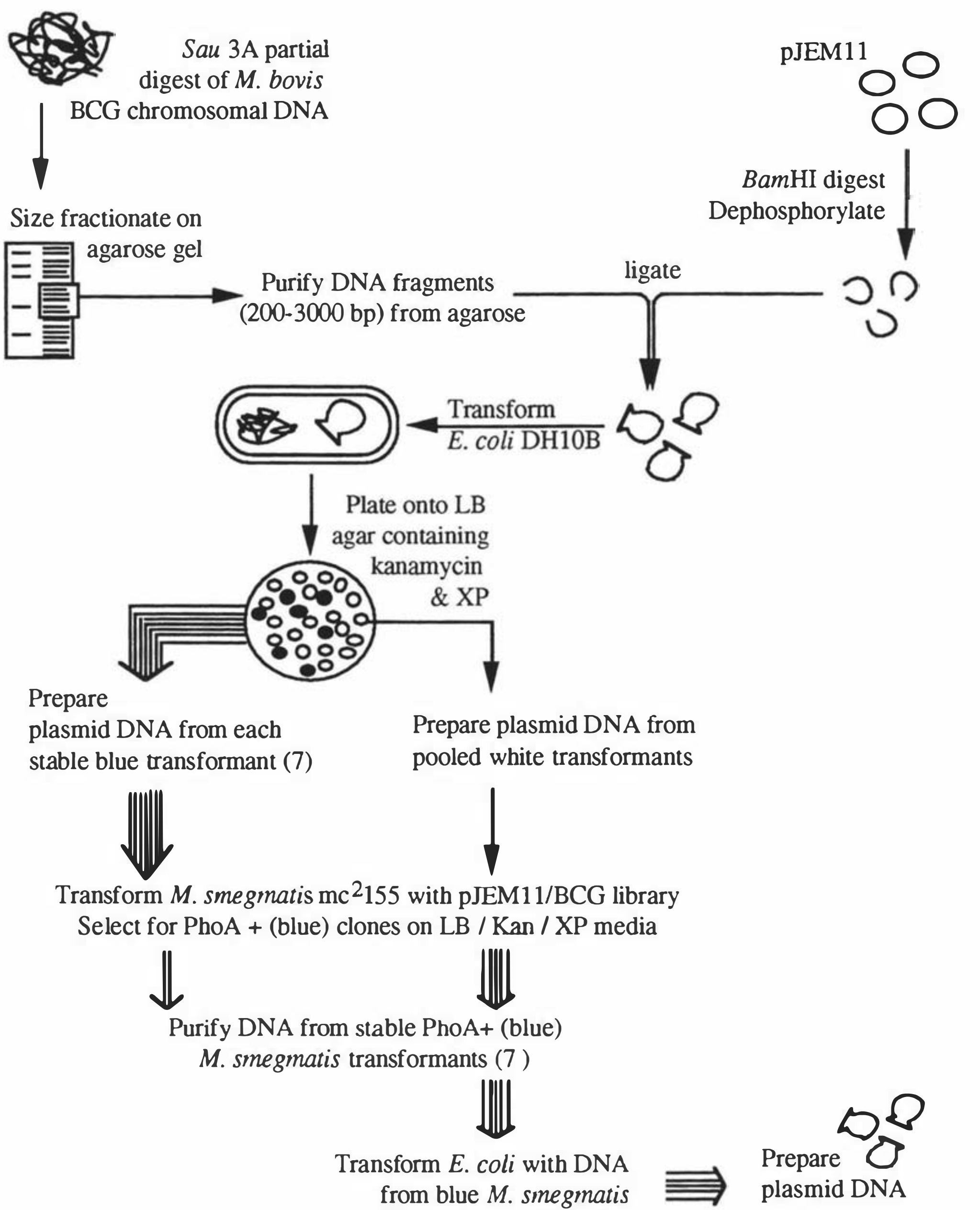



**Figure 3.2**

Construction of an *M. bovis* BCG library of *phoA* fusions using the vector pJEM11, and screening for clones expressing enzymatically active PhoA fusion proteins.

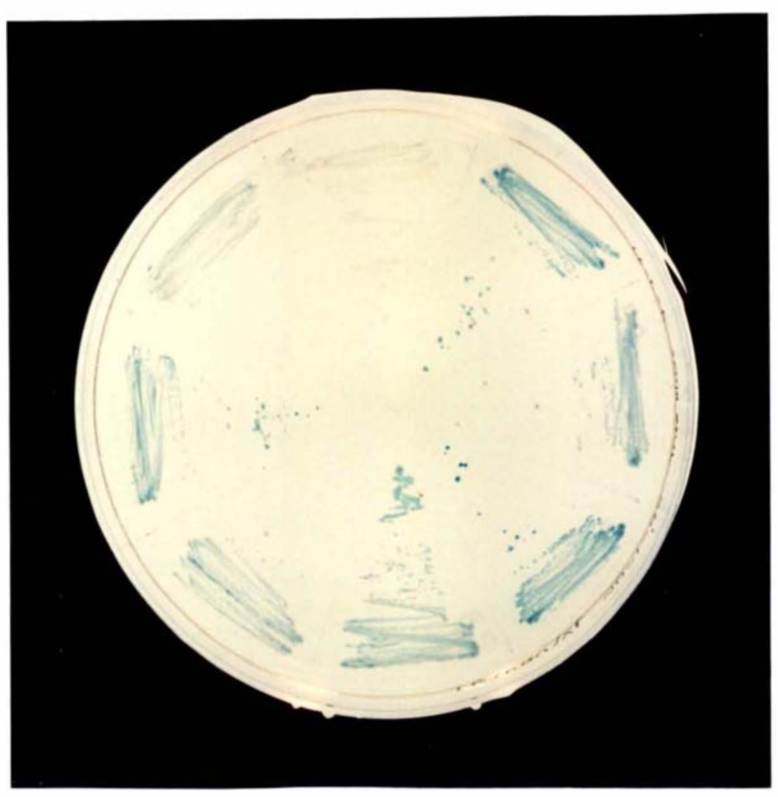


**Figure 3.3**

PhoA+ (blue) recombinant *E. coli* (plus a white negative control) restreaked for single colonies onto LB agar plates containing XP and kanamycin. Clockwise from top; *E. coli* containing the plasmids pJEM11, pE1 (pEM1), pE2, pE4 (pEM4), pE5, pE8, pE9, and pE14.

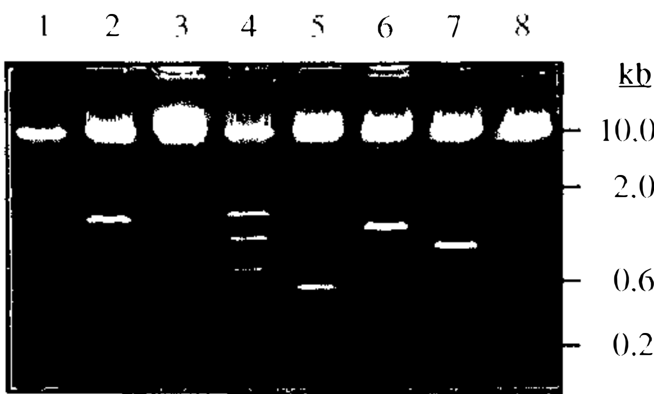



**Figure 3.4**

Restriction endonuclease analysis of plasmid DNA prepared from PhoA+ recombinant *E. coli* and a negative control. Plasmid DNA was digested with the enzymes *Kpn* I and *Apa* I to cleave the *M. bovis* BCG DNA insert from pJEM11. Fragments were run on an agarose gel alongside DNA size standards. Lane 1, pJEM11 control; lane 2, pE1; lane 3, pE2; lane 4, pE4; lane 5, pE5; lane 6, pE8; lane 7, pE9; and lane 8, pE14.

Plasmid DNA from the PhoA+ recombinant *E. coli* was used to transform *M. smegmatis* mc[2]155 cells. Recombinant *M. smegmatis* containing pE1 and pE4 turned blue after seven and four days, respectively. These plasmids were renamed pEM1 and pEM4 respectively. *M. smegmatis* was also transformed with the amplified pJEM11/*M. bovis* BCG genomic library using plasmid DNA prepared from pooled white *E. coli* transformants. Thirty-seven *M. smegmatis* transformants turned blue between five and ten days post-transformation. Figure 3.5 shows one strong and two pale blue colonies among the *M. smegmatis* transformants on day five. Transformants were restreaked for single colonies and five stable PhoA+ clones were isolated (*M. smegmatis* harbouring plasmids pM2, pM3, pM4, pM7 and pM21). DNA was purified from these recombinant *M. smegmatis* and transformed back into *E. coli* for further analysis. Plasmid DNA was isolated from the *E. coli* transformants and digested with *Kpn* I and *Apa* I. The *M. bovis* BCG DNA inserts ranged from 1.5 to 6.3 kb in length (Figure 3.6) with an average insert size of approximately 3.8 kb. Two plasmids, pM4 and pM7, had identical restriction patterns. This was confirmed using additional restriction enzymes, *Kpn* I and *Pst* I (data not shown). Therefore, pM7 was excluded from further studies. *M. smegmatis* cells were re-transformed with plasmid DNA purified from the *E. coli* transformants, and screening of the transformants on media containing XP verified that these clones expressed PhoA activity.

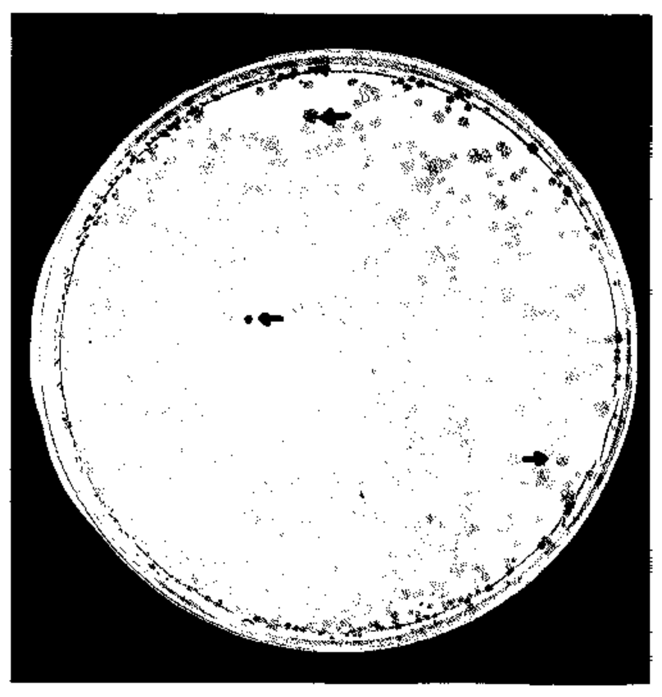


**Figure 3.5**

PhoA+ recombinant *M. smegmatis* turn blue in the presence of the chromogenic substrate, XP. *M. smegmatis* $mc^2155$ cells were transformed with the *M. bovis* BCG/pJEM11 library which had been amplified in *E. coli.* Five days after transformation, the first clones turned blue (as marked by arrows).

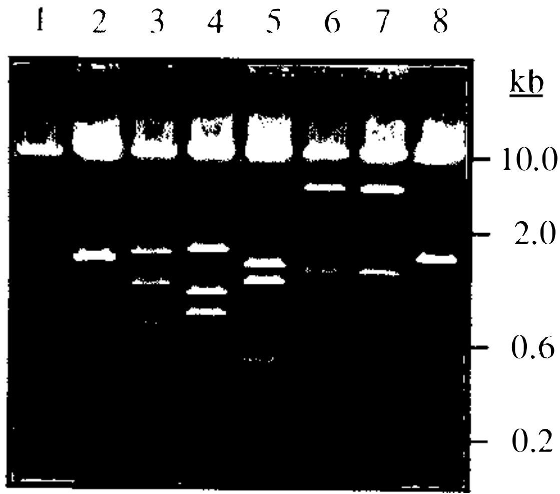



**Figure 3.6**

Restriction endonuclease analysis of plasmids that confer a PhoA+ phenotype on *M. smegmatis.* Plasmid DNA was purified from *E. coli* and digested with the enzymes *Kpn* I and *Apa* I to cleave the *M. bovis* BCG DNA insert from pJEM11. Fragments were run on an agarose gel alongside DNA size standards. Lane 1, pJEM11 control; lane 2, pEM1; lane 3, pEM4; lane 4, pM2; lane5, pM3; lane 6, pM4; lane 7, pM7; and lane 8, pM21.

### 3.3.2 Strain distribution of genes encoding exported proteins

Phenotypic heterogeneity among daughter strains of *M. bovis* BCG has long been recognised, and, for example, strains can be subdivided according to the level of expression of the secreted protein MPB70 (Harboe and Nagai, 1984; Abou-Zeid *et al.*, 1986). Differences between *M. bovis* BCG strains at the genomic level have also been noted. It has been shown that the genes encoding the secreted proteins MPB64 (Li *et al.*, 1993) and ESAT-6 (Harboe *et al.*, 1996) are absent in some *M. bovis* BCG daughter strains. In addition, gross genomic differences between *M. bovis* and *M. bovis* BCG have recently been identified (Mahairas *et al.*, 1996). Therefore, the strain distribution of the newly identified *M. bovis* BCG sequences from this study was examined using Southern blot analysis.

Genomic DNA was extracted from strains of *M. bovis* and *M. bovis* BCG. The DNA was digested with *Sal* I, and after electrophoresis was blotted onto nylon membranes. Insert DNA from plasmid constructs (pEM1, pE2, pEM4, pE5, pE8, pE9, pE14, pM2, pM3, pM4 and pM21) were purified from the pJEM11 vector using *Kpn* I, and *Apa* I or *Pst* I. The DNA inserts were labelled with [$\alpha^{32}$P]-dCTP and used to probe restriction digests of *M. bovis* and *M. bovis* BCG genomic DNA. The probes hybridised to all strains tested and to fragments of identical size in each strain indicating the genes were present and conserved among the strains tested. The result of a representative Southern blot is shown in Figure 3.7.

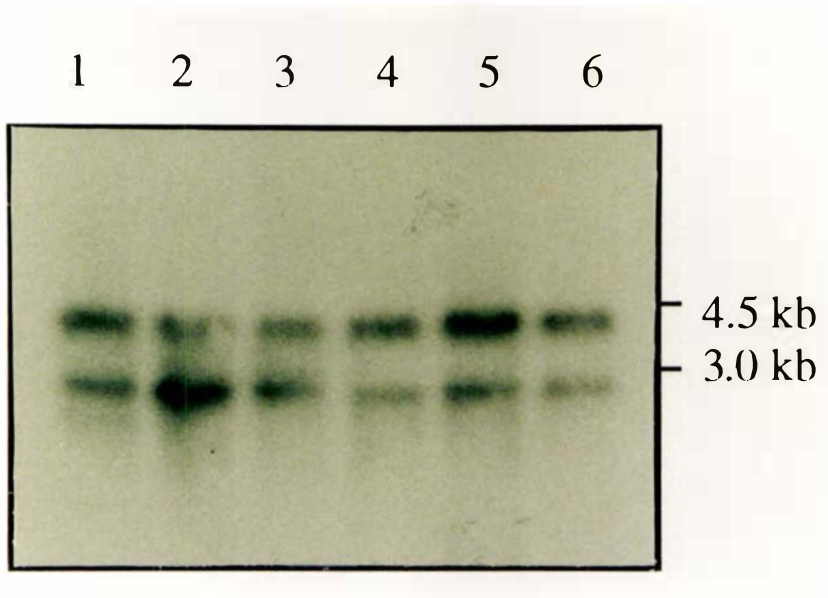



**Figure 3.7**

Southern blot of *Sal* I digested *M. bovis* and *M. bovis* BCG genomic DNA probed with radiolabelled insert DNA from pM3. Lane 1, *M. bovis* BCG Pasteur; lane 2, *M. bovis* BCG Tokyo; lane 3, *M. bovis* BCG Russia; lane 4, *M. bovis* (KML); lane 5, *M. bovis* ATCC 19210; and lane 6, *M. bovis* ATCC 35725.

### 3.3.3 Partial sequencing of DNA inserts encoding exported proteins

Several *M. tuberculosis*, *M. bovis* and *M. bovis* BCG culture filtrate antigens have been characterised through cloning and sequencing of their coding genes (refer to Table 1.1, Chapter 1). The majority of these are actively secreted from the cell and analysis of their N-terminal amino acid sequences reveals features characteristic of the signal peptides required for protein export. Exceptions include L-alanine dehydrogenase (Andersen *et al.*, 1992a), superoxide dismutase (Zhang *et al.*, 1991), ESAT-6 (Sφrensen *et al.*, 1995), and Des (Jackson *et al.*, 1997), all of which appear to be actively secreted across the cell membrane in the absence of a recognisable signal peptide.

The junctions between *phoA* and selected *M. bovis* BCG DNA inserts were sequenced to identify the ORFs responsible for expression of the PhoA fusions. The deduced amino acid sequences of selected PhoA fusions were also examined for putative signals directing protein export. DNA and protein databases were searched using the BLAST algorithm (Altschul *et al.*, 1990) to identify similarities between the insert DNA and previously determined sequences. Insert DNA sequence data are presented in Appendix 3.

Identification of a *phoA*-fusion with similarity to a putative 1.4 kb gene from *M. leprae*

Sequencing of the insert DNA of pM21 revealed an ORF in frame with *phoA*. When compared in database searches, the ORF showed approximately 75% similarity to the nucleotide sequence of an uncharacterised *M. leprae* ORF (coding sequence 28,586-29,995) from cosmid B1756 (Genbank accession # U15180) (Figure 3.8A). The putative start of the pM21 ORF lies 554 or 560 bases upstream from the point of fusion to *phoA* depending on whether the translation initiation codon is AUG or GUG. The amino acid sequences of the putative *M. leprae* 469 amino acid product (u1756V) and the *M. bovis* BCG homologue were compared (Figure 3.8B). Both proteins have the hydrophobic stretch of amino acids near their N-termini that is characteristic of signal

peptides (Pugsley, 1993). The *M. leprae* and *M. bovis* BCG putative signal peptides have several features in common with lipoprotein signal peptides; (a) a cysteine residue immediately after a potential signal peptidase cleavage site, (b) hydrophobic residues within the "N" domain, and (c) lack of a glycine or proline residue that is typically found at or around position -6 with respect to the cleavage site. A homology search using the BLAST algorithm (Altschul *et al.*, 1990), combined with the BEAUTY algorithm for predicting structural motifs (Worley *et al.*, 1995), identified prokaryotic membrane lipoprotein lipid attachment consensus sites in both the *M. leprae* and *M. bovis* BCG sequences, suggesting that these putative proteins might be exported lipoproteins. A putative ribosomal binding site was observed upstream of the *M. bovis* BCG ORF.

(A)

```
                                                        SD
pM21 ORF          CGGGATCTGCTGGCCGCGTCTGGCGATCTGAAGCCCGGACGAGAGGCAAAT      50
M. leprae ORF     ::ATC:GCTGAC::::C:GGC:ATC:GGC:CCTTGACGG:::G::AGG:G       50

                  GTGGTCATGAGTCGCGGGCGGATACCGAGGCTGGGCGCTGCCGTACTGGT      100
                  ::::GG::::::::G---::CG:C:ACC:AGC:::TA:AAT:A::::T:C       97

                  GGCGTTGACGACCGCGGCGGCG------GCGTGCGGGGCC  /  ATCGCT     572
                  A::T:::::CCTT:::T:A:T:GTGTTG::C::T::::::  /  G:G:::     575

                  GAGGCGGCCCGGCTGCACGCGGCGGGCGAGCCTAGCTGGATC / phoA       614
                  ::::::::T:::T::::T:::::C:::::::::C::::::::T             617
```

(B)

```
                  Start                   ↓
M. bovis BCG      VVMSRGRIPRLGAAVLVALTTAAA--ACGADSQGLVVSFYTPATDGATFT       48
M. leprae         ::V::-:VH:A:TII:A:::L:SVVL::::GGDQ::I::::::SED::::       49

                  AIAQRCNQQFGGRFTIAQVSLPRSPNEQRLQLARRLTGNDRTLDVMALDV       98
                  EV:R::TE::D:::A:QH:::::::D:::::::::::::K::S:::::M::      99

                  VWTAEFAEAGWALPLSDDPAGLAENDAVADTLPGPLATAGWNHKLYAAPV      148
                  ::::::::::::T::::E:::::::P::IV:::::::::::T:KR:::::::    149

                  TTNTQLLWYRPDLVNSPPTDWNAMIAEAARLHAAGEPSWI / PhoA        188
                  ::::::::::::T:::DQ::G:::G:V::::::::::::::::            189
```

**Figure 3.8**

Comparision of ORFs from *M. leprae* and the *M. bovis* BCG DNA insert from pM21. Divergent nucleotides and amino acids are shown only for *M. leprae*. The upstream nucleotide sequences are shown and the ORF as far as the junction of the *M. bovis* BCG DNA insert and *phoA* in (A). The putative initiation codon (GTG) is shown in bold and is preceeded by a putative Shine and Dalgarno (SD) sequence. The putative amino acid sequences are shown using the single letter code (B). The arrow indicates a putative signal peptidase cleavage site.

Identification of an ORF with homology to a family of high-temperature-requirement serine proteases

The insert DNA sequences of pM2 was partially determined on one strand. Database searches using the BLAST algorithm with the pM2 insert revealed regions of high similarity to the *htrA* gene of *M. tuberculosis* (69% over 150 bases) (Wu *et al.*, 1997), and to an ORF within an *M. leprae* cosmid (B1756, Genbank accession # U15180; coding sequence 21,036-22,637), named *htrA*. (64% over 150 bases). The database search also identified lesser similarity to *htrA* genes from *E. coli*, *Salmonella typhimurium*, *Brucella abortus*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*.

The reading frame of the pM2 *phoA*-fusion was determined and the predicted amino acid sequence of the pM2-encoded peptide was also similar to the deduced protein sequence of *M. tuberculosis* HtrA (Figure 3.9). The *M. bovis* BCG putative HtrA protein contains the motif Gly-Asn-Ser-Gly-Gly-Ala-Leu which matches the consensus sequence for the catalytic domain of serine proteases (Boucher *et al.*, 1996). The *htrA* family of genes code for exported, stress-response serine proteases that function by degrading abnormally folded proteins, thus preventing their accumulation to toxic levels within cells (Davies and Lin, 1988; Strauch *et al.*, 1989). The *E. coli htrA* gene is identical to *degP*, a periplasmic space protease (Lipinska *et al.*, 1989).

(A)

```
M. bovis BCG (pM2)        ----GCTCTCAACCGTCCAGTGTCGACGACCGGCCAGGCCGGCAACCAGA     46
M. tuberculosis htrA      CAGC::G::AC::::C::C::TC::TT:T:G:::G:::G:TCTG::ACCG   1150

                          ACACCGTGCTGGACGCCATTCAGACCGACGCCGCGATCAACCCCGGTAAC     96
                          :::::::CA:T::::A:::::::::::::::T::::::::A:::::::   1200

                          TCCGGNGGCGCGCTGGTGAACATGAACGCTCAACTCGTCGGAGTCAACTC    146
                          :::::C::TC::::AA:CG:::::G:T:CC::GG:GA:T::CA::::CA:   1250

                          GGCCATTGCCACGCTGGGCGCGGACTCAGCCGATGCGCAGAGCGGCTCGA    196
                          C:::GG:AAGTCA:::---T::::TAGC:::AGC----------------   1281

                          TCGGTCTCGGTTTTGCGATTCCAGTCGACCAGGCCAAGCGCATCGCCGAC    246
                          --::G::G::C::::::::C::G:::A::G::ATG::ATTGG:G::AA:T   1329

                          GAGTTGATCAGCACCGGCAAGGCGTNACATGCCTCCCTGGGTGT------    290
                          TCTC::::::AAGA:::A:::ATCGTG:::C:GA:GT::::CA:CAGCAC   1379

                          ---------------------------------GCAGGTGACCAATGACA    307
                          CCGGTCAGTAAGCAACGCGATCGCGTCGGGCGC:::::::G::::::TA:   1429

                          AAGACACSCCGGGCKCCAAGATC / phoA                        330
                          :G:CGGGAAGTCC:G:GC:::AG                              1452
```

(B)

```
M. bovis BCG (pM2)        ------------------ALNRPVSTTGQAGNQNTVLDAIQTDAAINPGN     32
M. tuberculosis HtrA      AVGAPLGLRSTVTQGIVS::H:::PLS:EGSDTD::I:::::::S::H::    400

                          SGGALVNMNAQLVGVNSAIATLGADSADAQSGSIGLGFAIPVDQAKRIAD     82
                          :::P:ID:D::VI:I:T:GKS:-S:::S------::::::::NEM:LV:N    443

                          ELISTGKAXHASLGV-------------QVTNDKDTPGXRI / PhoA      107
                          S::KD::IV:PT::ISTRSVSNAIASGA::A:V:AGSPAQKGGILENDVI    493
```

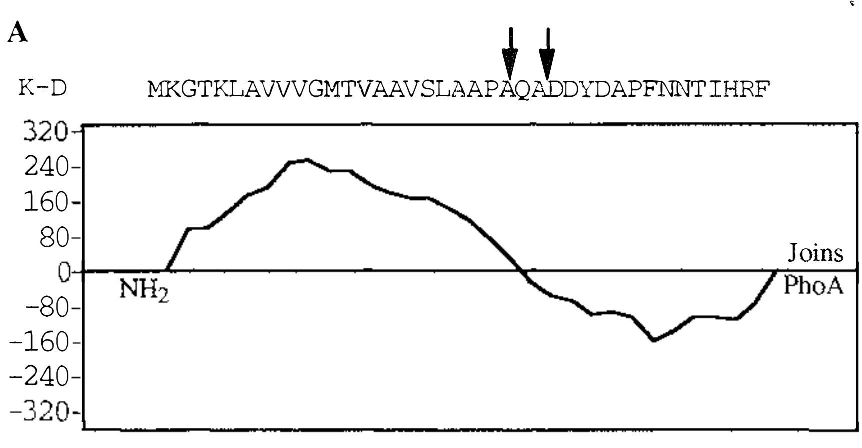
**Figure 3.9**

The region of similarity between the pM2 *M. bovis* BCG DNA insert and the *M. tuberculosis htrA* gene at (A) the nucleotide and (B) amino acid level. Spaces were introduced using GeneWorks to maximise the alignment. Divergent nucleotide and amino acids are shown for only *M. tuberculosis*. Ambiguous nucleotides: N = G, A, T or C; S = G or C; K + G or T. Amino acids are shown using the single letter code. X represents an unknown amino acid, not a stop codon. The residues in bold correspond to the consensus serine protease catalytic domain.

Identification of PhoA-fusions containing putative N-terminal signal peptides

Sequencing of the DNA inserts from pE2 and pEM4 revealed ORFs in frame with *phoA*. Database searches using the pE2 and pEM4 insert DNA sequences revealed 100% identity with uncharacterised sequences in the *M. tuberculosis* cosmids cSCY24G1 (EMBL accession # Z83858; nucleotide bases 8,977-9,132), and SCY25D10 (EMBL accession # Z95558; nucleotide bases 36,866-36,616), respectively. Translation of the pE2 and pEM4 ORFs revealed potential peptides characteristic of gram-negative signal peptides (Chapter 1.4.3). The deduced amino acid sequence of each PhoA fusion protein is shown in Figure 3.10 along with a hydrophobicity plot indicating the position of the putative signal peptides. The N-terminal region of the PhoA fusion protein encoded by pEM4 (Figure 10A) is typical of a standard signal peptide (Pugsley, 1993). It contains a short hydrophilic region (N domain: amino acids 1 to 5), followed by a hydrophobic core (H domain) of 16 residues (amino acids 6 to 21). This putative signal peptide also contains two turn-inducing amino acids; a glycine within the putative H domain and a proline at the start of the carboxy-terminal region (C domain); and two potential cleavage sites (alanine-X-alanine boxes). The predicted N-terminus of the pE2-encoded PhoA-fusion protein (Figure 3.10B) has all the features characteristic of a lipoprotein signal peptide; (a) the region close to the putative lipoprotein cleavage site (LSG↓C) closely matches the lipoprotein signal peptidase 'consensus' sequence (L,V,I)(A,S,T,G)(G,A)↓C, (b) a high incidence of turn-promoting residues (DGNPS) immediately downstream of the putative cleavage site, and (c) the core of highly hydrophobic amino acids (18 residues; amino acids 7 to 24) that is common to all signal peptides (von Heijne, 1989).

**A**




**B**

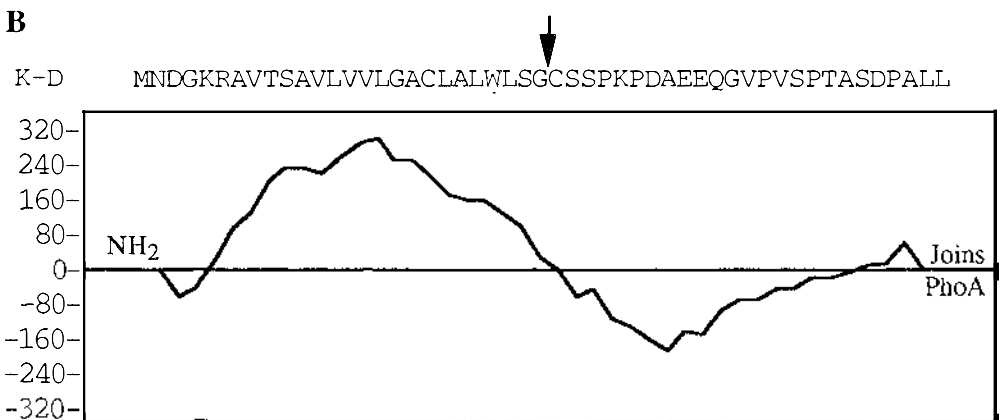



**Figure 3.10**

Hydrophobicity analysis (Kyte-Doolittle) plots of deduced amino acid sequences from ORFs in frame with *phoA*. Hydrophobic amino acids are represented by positive values. The amino acid sequences are encoded by *M. bovis* BCG DNA inserts from (A) pEM4 and (B) pE2. Putative signal peptide cleavage sites are indicated by arrows. The pE2-encoded PhoA fusion protein closely resembles a lipoprotein consensus sequence, whereas the pEM4-encoded sequence conforms to the rules for a standard signal peptide.

PhoA fusion proteins encoded by pE5, pE9 and pE14 lack recognisable signal peptides

DNA sequencing of pE9 and pE14 revealed overlapping insert sequences containing a single ORF fused in frame with *phoA* (see Appendix 3). The ORF in pE9 extends for a further 60 nucleotides (equivalent of 20 amino acid residues) downstream of the point where pE14 joins to *phoA*. These sequences are identical to a 528 base uncharacterised ORF within the *M. tuberculosis* cosmid Y78 (EMBL accession # Z77165; coding sequence 3,404-3,934). A putative Shine and Dalgarno sequence was located upstream of the ORF. Analysis of the translated product of the pE9 and pE14 ORF revealed no hydrophobic stretches of amino acids that might function as a signal peptide.

The complete DNA insert from pE5 was sequenced on both strands and databases were searched for similarities to known sequences. Nucleotides 1 to 351 of the 509 base pair insert were identical to a sequence located within the *M. tuberculosis* cosmid SCY20G9 (EMBL accession # Z77162; nucleotides 33,270-33,620). In contrast, nucleotides 352-509 showed no similarity to any database sequences. A *Sau* 3AI site exists at the point where the identity to the *M. tuberculosis* cosmid ends, suggesting that two *Sau* 3A fragments were ligated together during construction of the *M. bovis* BCG library. This was supported by the observation that oligonucleotide primers, designed to the flanking regions of the *Sau* 3A site in the *M. tuberculosis* cosmid sequence, yielded a PCR amplification product of the predicted size (based on the *M. tuberculosis* sequence) from *M. bovis* BCG genomic DNA (data not shown). An ORF was identified that was in frame with *phoA* and spanned the *Sau* 3A junction of the hybrid insert. Hydrophobicity analysis of the translated ORF revealed no recognisable signal peptide.

### 3.3.4 Detection of PhoA fusion proteins from recombinant *E. coli*

The molecular weights of the PhoA fusion proteins were determined by probing lysate samples of recombinant *E. coli* with rabbit anti-PhoA antibodies. Lysate samples of seven PhoA+ *E. coli* transformants and a non-recombinant control were prepared from 25 ml overnight cultures. Cells were washed twice by centrifugation and resuspended in 5 ml of buffer. The cells were lysed by sonication and the insoluble cellular debris removed by centrifugation. The protein concentration of each *E. coli* lysate sample was determined. Lysate samples were separated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with polyclonal antibodies raised against *E. coli* PhoA (Rockland, Pennsylvania). In each recombinant *E. coli* lysate sample, the antibody probe detected more than one protein (Figure 3.11). A protein band of 54 kDa was detected in each sample, including the *E. coli* control. Commercially purified PhoA was run on the gel as a positive control and also had an apparent molecular mass of 54 kDa. However, additional higher molecular weight bands were observed in the recombinant *E. coli* samples, ranging in size from 77 kDa (pE9) to a doublet of approximately 58 and 61 kDa in pEM1, pEM4, and pE5. The predicted and observed molecular weights of the PhoA fusion proteins were compared. The predicted molecular weight of the unprocessed pE9-encoded PhoA fusion protein is approximately 68 kDa, 9 kDa smaller than the observed fusion protein.

The predicted molecular weight of the unprocessed pE14-encoded PhoA fusion protein (~65 kDa) is also smaller than the observed molecular weight (~71 kDa). However, the 58/61 kDa doublet protein bands observed in the *E. coli* (pEM4) sample are similar to the predicted mature and unprocessed forms of the pEM4-encoded PhoA fusion protein. The observed molecular weight of the pE2-encoded fusion protein also matches that predicted for the unprocessed protein. The existence of multiple forms of each PhoA fusion protein suggests they may have been post-translationally modified, possibly by proteolytic degradation. No protein bands of less than 54 kDa were observed, indicating that proteolysis appeared to be limited to the *M. bovis* BCG-encoded polypeptide.

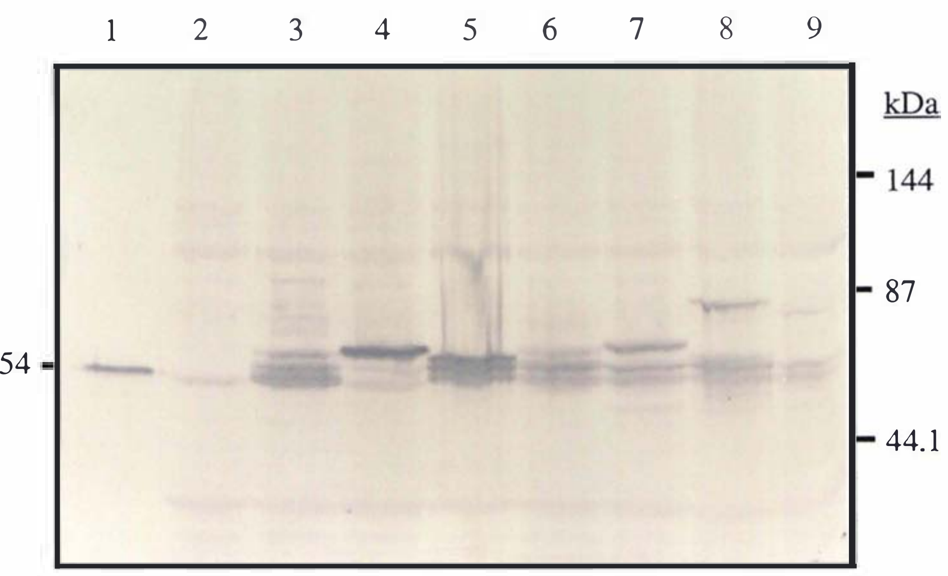



**Figure 3.11**

Western blot analysis of PhoA fusion proteins from recombinant *E. coli* lysate samples prepared from 25 ml cultures. Lane 1, commercial bacterial PhoA. Lane 2, non-recombinant *E. coli* DH10B lysate (control). Lanes 3-9 contain cell lysate samples of recombinant *E. coli* harbouring the following plasmids: lane 3, pEM1; lane 4, pE2; lane 5, pEM4; lane 6, pE5; lane 7, pE8; lane 8, pE9; and lane 9, pE14.

### 3.3.5 Detection of PhoA fusion proteins from recombinant *M. smegmatis* by Western blot analysis

Signal peptides are required for the translocation of exported proteins across the cytoplasmic membrane. In Gram-negative *E. coli*, alkaline phosphatase is exported to the periplasmic space which lies between the cell's inner and outer membranes. However, the Gram-positive bacterium *Mycobacterium smegmatis*, has a single cell membrane beneath a thick, lipid-rich cell wall. This raises the question as to the fate of PhoA fusion proteins in this Gram-positive host. The exported fusion proteins may be secreted through the cell wall into the culture medium or remain associated with the cell membrane or cell wall. To determine the location of the PhoA fusion proteins from blue (PhoA+) recombinant *M. smegmatis*, cell lysates and CF were subjected to Western blotting using anti-PhoA antibodies.

Recombinant *M. smegmatis* colonies were scraped from agar plates and resuspended in a small volume of buffer. The cells were pelleted in a microcentrifuge and the supernatant (extracellular wash) removed. The cell pellets were resuspended in buffer and lysed by sonication. The protein concentration of recombinant *M. smegmatis* lysates and extracellular wash samples was determined. Quantitative PhoA assays revealed that <1% of the PhoA activity was present in the extracellular wash fraction (data not shown). Lysate samples were size fractionated by SDS-PAGE immediately following sonication to minimise protein degradation. The Western blots were probed with anti-PhoA antibodies. Three common protein bands were detected in lanes containing lysate samples from each recombinant *M. smegmatis* clone with a PhoA+ phenotype (Figure 3.12). The higher molecular weight band in each lane was approximately 54 kDa, the same apparent molecular weight as purified PhoA. The lower molecular weight bands were of similar size to breakdown products observed in the lane containing a commercial PhoA sample. In *M. smegmatis* samples containing pEM1, pEM4 and pM2 the anti-PhoA antibody also bound to larger proteins, suggesting that the antibody was detecting

full length PhoA fusion products. The PhoA fusion protein encoded by pEM1 had a molecular weight of approximately 90 kDa. Two additional *M. smegmatis* (pEM4) proteins were detected; one of approximately 60 kDa, the predicted molecular weight of the pEM4-encoded fusion protein, and another of approximately 100 kDa. In *M. smegmatis* (pM2), a protein band of approximately 95 kDa was also observed.

In addition to the plate scraping procedure, CFs from liquid cultures of recombinant *M. smegmatis* were also analysed by Western blotting to determine if any of the fusion proteins was secreted into the culture medium. Culture filtrate and lysate samples were prepared from 1 L cultures of *M. smegmatis* (pJEM11) and (pM21) and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with anti-PhoA antibodies to detect the fusion proteins expressed by *M. smegmatis* (pM21). The antibody detected three weak protein bands of the similar molecular weight in the *M. smegmatis* (pM21) CF and lysate sample (Figure 3.13). These bands were absent from both *M. smegmatis* (pJEM11) control samples. This indicated that the proteins were encoded by the insert DNA of pM21. However, the visualised proteins were of approximately equal or lower molecular weight compared to the commercial PhoA sample. This result suggests that there may be proteolytic degradation of the fusion protein encoded by pM21.

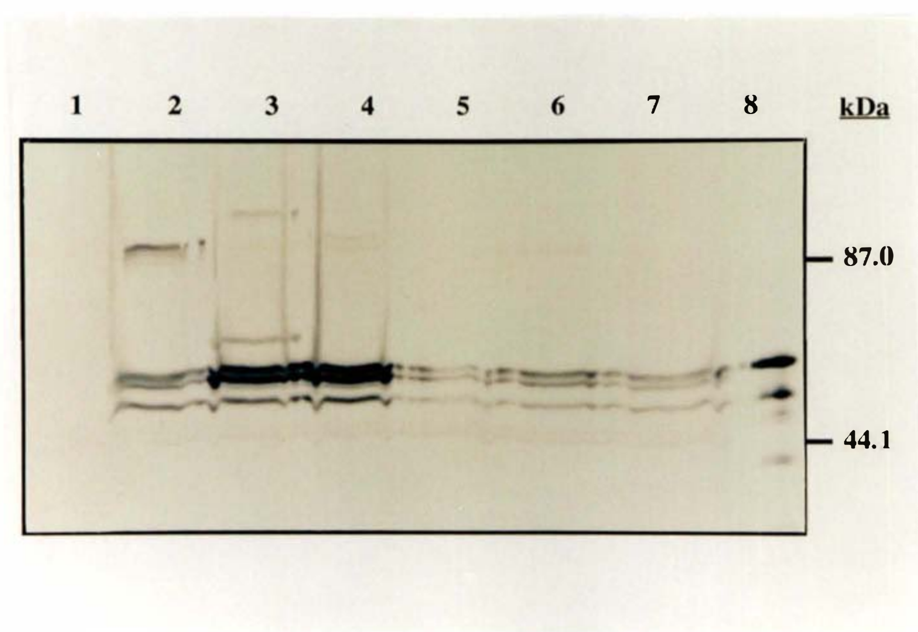



**Figure 3.12**

Western blot analysis of PhoA fusion proteins from recombinant *M. smegmatis* lysate samples prepared by scraping colonies from solid media. Lanes 1-7 contain lysate samples of recombinant *M. smegmatis* harbouring the following plasmids. Lane 1, pJEM11 (control); lane 2, pEM1; lane 3, pEM4; lane 4, pM2; lane 5, pM3; lane 6, pM4; and lane 7, pM21. Lane 8 contains a commercial bacterial PhoA sample.

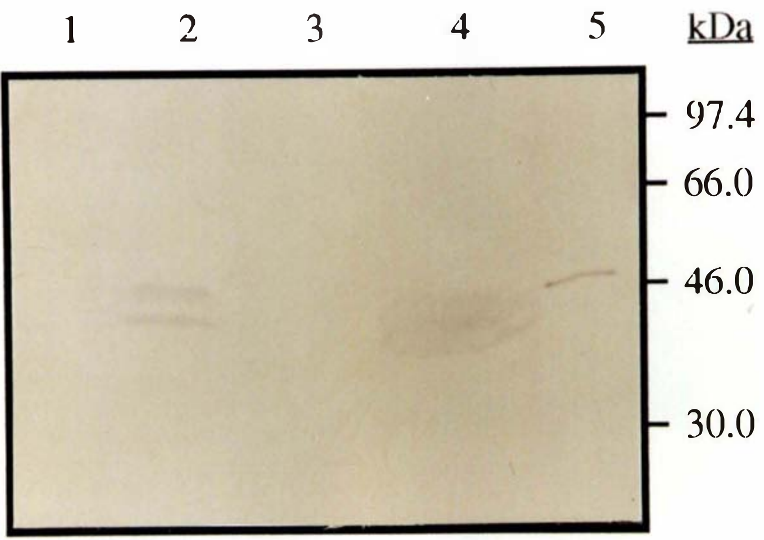



**Figure 3.13**

Western blot analysis of PhoA fusion proteins from *M. smegmatis* (pM21) lysate and CF samples prepared from 1L cultures. Lane 1, *M. smegmatis* (pJEM11) CF; lane 2, *M. smegmatis* (pM21) CF; lane 3, *M. smegmatis* (pJEM11) lysate; lane 4, *M. smegmatis* (pM21) lysate. Lane 5, commercial bacterial PhoA sample.

### 3.4 Discussion

In this study, an *M. bovis* BCG library of *phoA* fusions was constructed using pJEM11 and the DNA sequences encoding exported proteins were analysed. The gene library was transformed and analysed in both *M. smegmatis* and *E. coli*. Approximately 8000 *E. coli* transformants were obtained upon plating out 7.5% of the library, 14 of which expressed enzymatically active PhoA fusion proteins, as indicated by their blue colour on media containing XP. Twelve of the pJEM11/*M. bovis* BCG plasmids prepared from blue *E. coli* transformants conferred a PhoA$^-$ phenotype on *M. smegmatis*. One possible explanation is that these PhoA-fusions are expressed and translocated across the cell membrane of *M. smegmatis* but they are unable to assume a conformation necessary for enzyme activity. Alternatively, they may contain cryptic export signals that are not recognised by the *M. smegmatis* export machinery. An additional 37 blue (PhoA+) *M. smegmatis* clones were observed following transformation with plasmid DNA prepared from the pooled white *E. coli* transformants. This result is consistent with previous studies (Clark-Curtiss *et al.*, 1985; Das Gupta *et al.*, 1993; Bashyam *et al.*, 1996) which have shown that *M. smegmatis* is a more permissive host for expression of *M. bovis* BCG genes than *E. coli*.

*M. bovis BCG* DNA inserts from plasmids expressing enzymatically active PhoA fusion proteins were partially sequenced and used to search databases for similarities to known genes. Database searches revealed similarities to a family of high temperature requirement (HtrA) serine proteases (pM2), a putative 1.4 kb lipoprotein gene from *M. leprae* (pM21), and several uncharacterised *M. tuberculosis* ORFs. The *M. bovis* BCG DNA insert of plasmid pM2 showed similarity to a *htrA* gene of *M. tuberculosis* (Wu *et al.*, 1997) and *htrA* genes from several different bacterial species (Lipinska *et al.*, 1988; Johnson *et al.*, 1991). The HtrA proteins belong to a family of stress-response serine proteases, and multiple *htrA* genes have been identified within the same species (Waller and Sauer, 1996). On the basis of the level of similarity of the *M. bovis* BCG pM2 insert

sequence to the *M. tuberculosis htrA* gene (69% over 150 bases), it is likely that multiple *htrA* genes are also present in mycobacteria. HtrA proteases prevent toxic accumulation of incorrectly folded proteins, including heat denatured and oxidatively damaged proteins (Davies and Lin, 1988; Strauch and Beckwith, 1988; Boucher *et al.*, 1996). The *htrA* genes of *B. abortus* and *S. typhimurium* have been directly implicated in bacterial virulence and survival within macrophages (Johnson *et al.*, 1991; Elzer *et al.*, 1994). It is possible that the product of the *M. bovis* BCG putative *htrA* gene identified in this study may also play a role in bacterial survival within macrophages. The mycobacterial HtrA-like proteases may contribute to defence against toxic effects of reactive intermediates generated by host macrophages. Further characterisation of the *M. bovis* BCG putative HtrA protein identified in this work is necessary to determine if this exported protein plays a role in intracellular survival.

The complete *M. bovis* BCG DNA insert of pM21 was sequenced and used to search DNA and protein databases. This *M. bovis* BCG insert was found to be highly similar to the nucleotide sequence of an uncharacterised *M. leprae* ORF contained within cosmid B1756. Sequence analysis of the putative 469 amino acid product (u1756V) and its *M. bovis* BCG homologue suggest that they are exported lipoproteins and contain consensus membrane lipid attachment sites (Pugsley, 1993; Worley *et al.*, 1995). If the putative *M. bovis* BCG protein is a lipoprotein, it is not necessarily present in a cell-associated form only, as the 19 kDa lipoprotein of *M. tuberculosis* is released into the culture medium (Fifis *et al.*, 1991). Western blot analysis of *M. smegmatis* (pM21) samples revealed multiple alkaline phosphatase-sized products in both the cell lysate and CF samples, suggesting that a soluble, secreted form of the putative *M. bovis* BCG lipoprotein may exist.

The remaining plasmids conferring a PhoA+ phenotype contained DNA sequences which showed no similarity to previously characterised genes. Two plasmids (pE2 and pEM4) encoded PhoA fusion proteins with consensus signal peptides. However, two of the

BCG DNA inserts (pE5 and pE14) encoded enzymatically active PhoA fusion proteins with no recognisable signal peptides. Neither fusion protein contains the hydrophobic core near the predicted N-terminus that is characteristic of a signal peptide. It is unclear how these gene fusions confer a PhoA+ phenotype on their *E. coli* hosts. One possibility is that cryptic export signals, located at the N-terminal region, facilitate interaction with the *E. coli* export machinery, allowing translocation across the cytoplasmic membrane. Protein secretion in the absence of a consensus signal peptide is known to occur in mycobacteria. The *M. tuberculosis* secreted proteins SOD (Zhang *et al.*, 1991), L-alanine dehydrogenase (Andersen *et al.*, 1992a), ESAT-6 (Sφrensen *et al.*, 1995), and Des (Jackson *et al.*, 1997) lack recognisable signal sequences and yet appear to be actively secreted from the cell. This suggests that a signal peptide-independent mechanism of protein export may exist in mycobacteria. These secreted proteins, when expressed in *E. coli* may function in a similar manner.

The PhoA fusion proteins expressed by the PhoA+ *E. coli* recombinants were identified by probing Western blots of cell lysates with anti-PhoA antibodies. Multiple protein bands were detected by the antibody in each recombinant *E. coli* sample, suggesting that they represent breakdown products of the full-length fusion proteins. Western blots of lysates and CFs from PhoA+ *M. smegmatis* recombinants using anti-PhoA antibodies also revealed products of a similar size to purified PhoA. In *E. coli* and *M. smegmatis*, PhoA hybrid proteins can be subjected to proteolysis, releasing alkaline phosphatase-sized degradation products (Manoil and Beckwith, 1985; Lim *et al.*, 1995). In *E. coli*, mutants unable to generate these breakdown products are defective in the periplasmic protease, DegP (Strauch and Beckwith, 1988). The DegP protein degrades unstable PhoA fusion proteins to yield smaller, conformationally stable products with alkaline phosphatase activity (Strauch and Beckwith, 1988). Exported fusion proteins also appear to be susceptible to proteolytic breakdown in *M. smegmatis* (Winter, pers. comm., 1996). The detection of multiple protein bands in *E. coli* and *M. smegmatis* in this study is consistent with a post-translational degradation of the PhoA fusion proteins.

It was noted that the molecular weight of the final breakdown product was similar to native alkaline phosphatase, suggesting that the fusion proteins had been degraded to the smallest stable form. However, it is possible that the screening system only detects those fusion proteins that can form stable and PhoA active proteins. The generation of unstable fusion proteins may lead to degradation which destroys PhoA activity. In this screening system, these would be recorded as PhoA- and thus would be indistinguishable from fusion proteins lacking export signals. It is unclear whether the degradation of fusion proteins is related to the primary polypeptide sequence. However, the fact that the *M. bovis* BCG moiety of the fusion proteins are different in their predicted amino acid composition suggests that conformation may be the main determinant for protein stability. Because of the instability associated with the fusion proteins identified in this study, the DNA inserts were used to screen for the full-length coding sequences in a cosmid library of *M. bovis*.

# Chapter 4:

# Identification Of Genes Encoding Exported Proteins In An *M. bovis* Cosmid Library

## 4.1 Abstract

A cosmid library of *M. bovis,* expressed in *M. smegmatis,* was screened for the presence of genes coding for exported proteins. In the first instance, the cosmid library was screened using PCR primers generated against several genes coding for previously characterised secreted antigens. Cosmids containing the genes coding for the 85-B antigen, PhoS, MPB64, MPB70, and ESAT-6 were identified. Western blot analysis of culture filtrate (CF) from the recombinant *M. smegmatis* containing the *mpb64* gene revealed that the *M. bovis* MPB64 antigen was expressed and secreted from *M. smegmatis.*

The DNA inserts from the PhoA+ clones (described in chapter 3) were used to screen the cosmid library to locate the complete genes coding for these exported proteins. Selected recombinant *M. smegmatis* containing cosmid genes coding for novel *M. bovis* exported proteins were grown in culture and the supernatants collected. The CFs were assayed for their ability to induce proliferation and IFN-γ-production from peripheral blood mononuclear cells (PBMCs) taken from *M. bovis* BCG-immunised cattle. Proliferative responses to the recombinant *M. smegmatis* (cosmid) CFs were highly variable. In contrast, the IFN-γ responses were more consistent, and two of the CF samples (cosmids 44 and 56) induced high levels of IFN-γ-production from PBMCs taken from two *M. bovis* BCG-immunised animals. Southern blot analysis, in conjunction with sequence data from one of the PhoA+ clones (pM21), revealed that cosmid 56 contained sequences with similarity to an *M. leprae* ORF encoding a putative exported lipoprotein.

## 4.2 Introduction

The identification of secreted *M. bovis* antigens and their coding genes constitutes a rational approach for development of a novel vaccine against bovine tuberculosis. Results from two dimensional gel electrophoresis of *M. bovis* BCG CFs have revealed a large number of secreted proteins which can stimulate proliferation of lymphocytes from *M. bovis* BCG-immunised cattle (Gulle *et al.*, 1995). This suggests that immunised cattle may provide a good model system to study antigen recognition and lymphocyte responses. A novel screening system has been described by Averill *et al.* (1993) using CFs derived from an *M. bovis* BCG cosmid library in *M. smegmatis* to identify antigens that induce T-cell proliferation from human tuberculin-positive donors. Thirty recombinant clones were tested and three produced secreted proteins with T-cell stimulatory activity (Averill *et al.*, 1993). A similar approach has been employed in this laboratory to identify cosmid clones coding for bovine T cell antigens (Carpenter *et al.*, 1995). Culture filtrates prepared from an *M. bovis* cosmid library in *M. smegmatis* were incubated with PBMCs taken from *M. bovis* BCG-immunised cattle to identify recombinants encoding antigens that stimulated proliferation and/or IFN-γ-production by T lymphocytes. The screening system was optimised to distinguish between *M. bovis* and *M. smegmatis* secreted proteins.

In the previous chapter, an *M. bovis* BCG library of PhoA fusions was screened for sequences coding for protein export signals. These sequences may represent partial genes fused to *phoA*. The protein analysis results indicated that the fusion polypeptides were partially degraded in *E. coli* and *M. smegmatis*. This chapter describes the screening of an *M. bovis* cosmid library with insert DNA from the PhoA+ clones to locate the complete genes. Culture filtrates from the recombinant *M. smegmatis* containing these exported protein genes were used to stimulate PBMCs from *M. bovis* BCG-immunised cattle to identify recombinants encoding T-cell antigens. Antigen recognition was measured by the proliferative response and IFN-γ secretion by PBMCs taken from immunised and control cattle.

## 4.3 Results

### 4.3.1 Restriction endonuclease analysis of DNA from selected cosmids

An *M. bovis* cosmid library was constructed at AgResearch, Wallaceville (Wilson and Collins, 1996) using an *E coli*-mycobacterial shuttle cosmid provided by Dr. W.R. Jacobs from the Albert Einstein College of Medicine, New York (Belisle *et al.*, 1991). Mycobacterial DNA fragments of 35-40 kb were cloned into the cosmid vector pYUB18 (Wilson and Collins, 1996).

The cosmid library was first transformed into *E. coli* by electroporation with selection for kanamycin resistance. Subsequent transformation of the library into *M. smegmatis* yielded 356 transformants. Cosmid DNA was extracted from ten randomly selected recombinant *E. coli*. The DNA was digested with *Eco* RI and the restriction patterns of each cosmid were compared (Figure 4.1). Ten different banding patterns were observed, suggesting that all the cosmids contained different DNA inserts. The common band at approximately 5 kb was found to correspond to the pYUB18 vector (data not shown). The cosmid inserts appeared to range from 25 to 35 kb in size, although they may be larger than this as some higher bands might represent more than one DNA fragment.

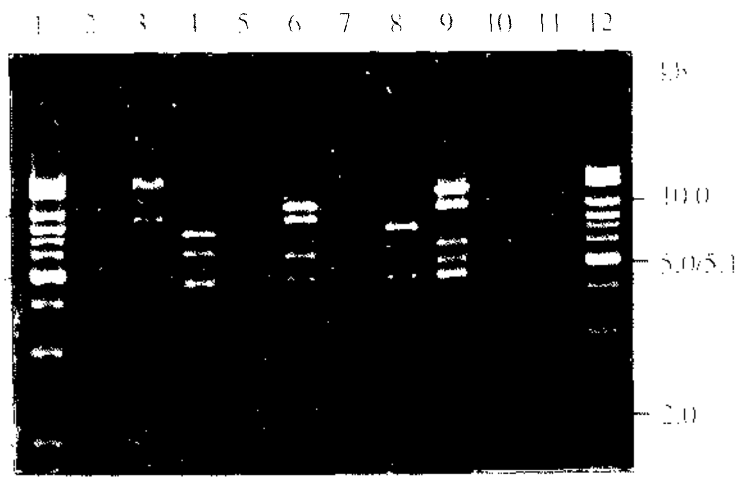



**Figure 4.1**

Restriction endonuclease analysis of cosmid DNA prepared from ten randomly selected recombinant *E. coli.* Cosmid DNA were digested with the enzyme *Eco* RI. Lanes 1 and 12, 1 kilobase extension ladder. Lanes 2 to 11 contain cosmids 15, 25, 30, 31, 83, 89, 106, 146, 155, and 164.

### 4.3.2 Screening of an *M. bovis* cosmid library for genes encoding exported proteins

After transformation of the library into *M. smegmatis*, the recombinants were patched onto Hybond-N+ Nucleic Acid Transfer Membrane discs (Amersham, Little Chalfont, England) and placed on top of 7H10 / OADC media containing kanamycin. Following *in situ* lysis of colonies, the membranes were probed with radiolabelled insert DNA from PhoA+ clones. In some experiments up to four insert DNA probes were pooled and used to screen the cosmid library in a single step. Figure 4.2 shows an autoradiograph of the cosmid library screened with pooled probes made from the DNA inserts of pEM1, pE2, pE5, and pE14. The DNA probes hybridised strongly to nine recombinant *M. smegmatis* (cosmids 2, 23, 45, 68, 86, 208, 209, 226, and 336). These cosmids were selected for secondary screening with the individual DNA probes.

The DNA was extracted from positive *M. smegmatis* recombinants and transformed into *E. coli*. The cosmid DNA was purified and digested with *Eco* RI and *Hin* dIII. Digestion of cosmids 68 and 226 produced identical restriction patterns and the use of a third restriction endonuclease confirmed that these cosmids were identical (data not shown). *Eco* RI digests of cosmids containing different inserts were run on agarose gels alongside *Sal* I-digested *M. bovis* (KML) genomic DNA and blotted onto a nylon membrane. The Southern blots were probed with [$\alpha^{32}$P]-dCTP-labelled insert DNA from the PhoA+ clones. A representative Southern blot of cosmids selected for secondary screening is shown in Figure 4.3 (probed with pE14 insert DNA). The pE14 insert DNA probe hybridised to restriction fragments from *M. bovis* genomic DNA, and cosmids 68 and 336. The hybridisation signal intensities differed due to uneven sample loading. The restriction fragments from cosmids 68 and 336 were different sizes, approximately 9.5 and 7.0 kb, respectively. In addition, restriction endonuclease analysis of these cosmids revealed common bands (data not shown). This indicated that these cosmids may contain overlapping sequences, and that the different sized bands on

the Southern blot may reflect the position of the junction between the insert DNA and the cosmid vector. Three recombinants that were positive in the primary screening (cosmids 45, 86, and 226) were excluded from this secondary screening for the following reasons. Cosmid 45 was unstable in *E. coli* and transformants did not grow in liquid cultures. Restriction endonuclease analysis revealed that cosmid 86 had lost its DNA insert, and cosmid 226 contained the same DNA insert as cosmid 68 (data not shown).

Cosmids identified by probing Southern blots with insert DNA from the PhoA+ clones are listed in Table 4.1. For each DNA insert probe, one hybridising cosmid was selected for study. Selected cosmids are indicated by an asterisk (*) in Table 4.1.

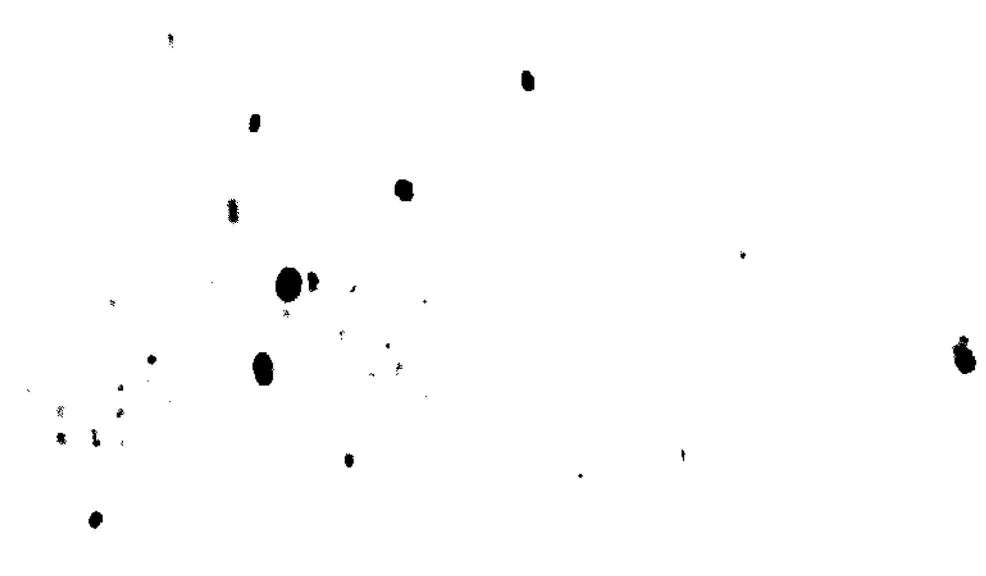


**Figure 4.2**

Screening the *M. bovis* cosmid library with insert DNA from PhoA+ clones. Following *in situ* denaturation, the membranes were probed with pooled radiolabelled DNA inserts from the PhoA+ clones pEM1, pE2, pE5, and pE14. DNA probe hybridised to recombinant *M. smegmatis* containing cosmids 2, 23, 45, 68, 86, 208, 209, 226, and 336.

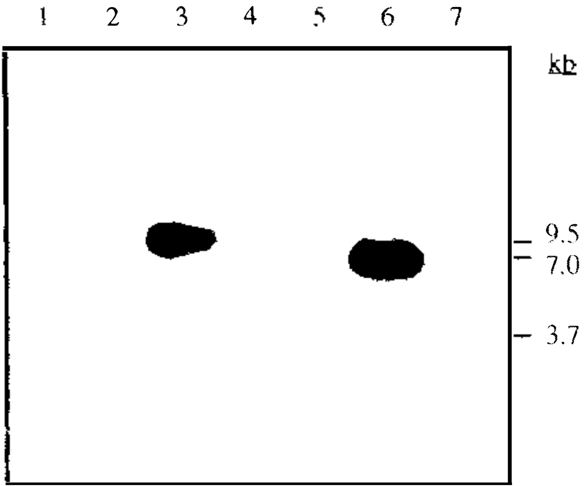



**Figure 4.3**

Secondary screening of the *M. bovis* cosmid library using radiolabelled insert DNA from pE14 as a probe. Cosmids which were positive in the primary screening were digested with *Eco* RI and the DNA fragments separated on an agarose gel. Lanes 1 to 6 contain cosmids 2, 23, 68, 208, 209, and 336 respectively. Lane 7, *Sal* I-digested M. bovis (KML) genomic DNA.

| PhoA+ clone | Size of *M. bovis* BCG DNA insert | Comments on DNA sequence of plasmid insert from PhoA+ clone | Cosmids hybridising to insert DNA probe |
|---|---|---|---|
| pEM1 | ~1.8 kb | Identical to sequence in *M. tuberculosis* cosmid cY13c5 | 23*, 209 |
| pE2 | 222 bp | Identical to ORF in *M. tuberculosis* cosmid cSCY24G1 | 2* |
| pEM4 | ~3.5 kb | Identical to ORF in *M. tuberculosis* cosmid SCY25D10 | 3*, 195, 277 |
| pE5 | 510 bp | Cloning rearrangements in insert | 44*, 155, 252 |
| pE8 | ~1.3 kb | No significant homology to known DNA sequences | 260* |
| pE9 | ~1.0 kb | Identical to ORF in *M. tuberculosis* cosmid Y78 | 68*, 226, 336 |
| pE14 | ~347 bp | Identical to bases 72 to 401 of pE9 insert | 68*, 226, 336 |
| pM2 | ~3.7 kb | Similar to *htrA* genes coding for a family of stress-response proteases | 275* |
| pM3 | ~3.3 kb | Identical to ORF in *M. tuberculosis* cosmid SCY13E12 | 48*, 98, 188 |
| pM4 | ~6.3 kb | Cloning rearrangements in insert | 32, 34, 51, 188, 196* |
| pM21 | 1521 bp | Similar to *M. leprae* ORF coding for a putative exported lipoprotein | 56*, 161 |

**Table 4.1**

Results from screening the *M. bovis* cosmid library with insert DNA from PhoA+ clones. Culture filtrates from *M. smegmatis* containing selected *M. bovis* cosmids* were prepared and used in immunoassays.

### 4.3.3 Identification of cosmids which contain genes coding for known secreted antigens

Almost thirty genes encoding *M. bovis* and *M. bovis* BCG culture filtrate proteins have been cloned and sequenced to date (Table 1.1, Chapter 1). Many of these code for proteins which are bovine T-cell antigens (Fifis *et al.*, 1994). The cosmid library was screened for the presence of the previously characterised secreted antigens MPB70, ESAT-6, 85-B and PhoS. PCR primers (Appendix 2A) were generated against the genes encoding these secreted proteins. DNA prepared from 200 recombinant *M. smegmatis* was pooled into groups of ten and subjected to PCR amplification. Each primer pair amplified a product the same size as the *M. bovis* genomic DNA product, from one of the DNA pools (data not shown). The individual cosmids from these pools were rescreened by PCR. Cosmids 3, 7, 15, and 79 were found to contain the genes *esat-6*, *mpb70*, *phoS*, and *85-B* respectively.

Selected cosmids which were positive from screening with the DNA inserts from PhoA+ clones (see Table 4.1) were also screened by PCR using primers (Appendix 2A) generated against a panel of ten genes (*85-A*, *85-B*, *85-C*, *mpb70*, *mpb64*, *esat-6*, *apa*, *sodA*, *phoS*, and the 19 kDa antigen gene). The amplified DNA fragments were compared in size to the products obtained following PCR of *M. bovis* genomic DNA with the same primers. With the MPB64 and ESAT-6 PCR primer pairs, products of identical size to the *M. bovis* PCR products were amplified from cosmid 260 and cosmid 3, respectively. Southern blot screening of the library with pEM4 revealed hybridising sequences in cosmids 3, 195 and 277. Each of these cosmids was subjected to PCR using the ESAT-6 primers. The results indicated that the *esat-6* gene is contained in cosmid 3, but is absent from cosmids 195 and 277 (Figure 4.4).

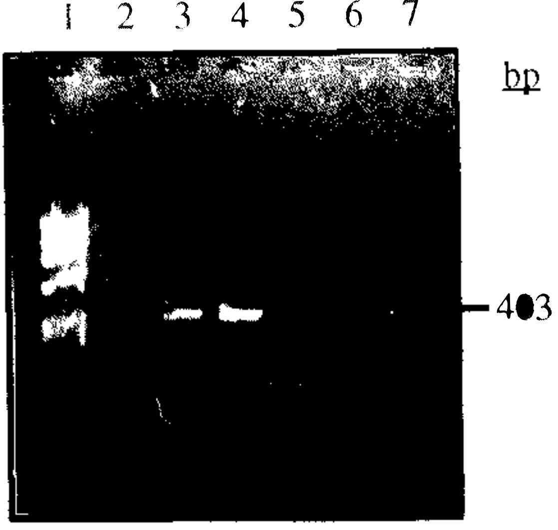



**Figure 4.4**

Amplification by PCR of a 403 base pair (bp) fragment from *M. bovis* genomic DNA and cosmid 3 using primer pairs generated against the *esat-6* gene. Cosmids 195 and 277 did not give an amplification product. Amplification products were run on an agarose gel alongside DNA size standards. Lane 1, $\phi$X174 DNA molecular weight markers; lane 2, no DNA control; lane 3, *M. bovis* genomic DNA; lane 4, cosmid 3; lane 5, cosmid 195; lane 6, cosmid 277; and lane 7, cosmid 44 (negative control).

### 4.3.4 Western blot identification of the *M. bovis* secreted antigen MPB64 in culture filtrate from recombinant *M. smegmatis*

Western blot analysis was carried out on a recombinant *M. smegmatis* CF sample to determine if *M. bovis* proteins could be synthesised and secreted into the culture medium from *M. smegmatis.* The results from the PCR experiments revealed that the gene coding for the secreted protein MPB64 was located on cosmid 260. Culture filtrates were prepared from *M. smegmatis* (cosmid 260), and a randomly selected recombinant (*M. smegmatis* containing cosmid 44) as a negative control. Culture filtrate was also prepared from *M. bovis* (strain KML). The proteins in each CF sample were separated by SDS-PAGE in duplicate. One half of the gel was silver-stained to ensure even sample loading (data not shown), and the duplicate samples were transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal antibodies raised against MPB64. The antibody probe bound to a protein of approximately 26 kDa in CF from *M. smegmatis* (cosmid 260) and *M. bovis* (Figure 4.5). An additional protein of approximately 22.5 kDa was observed in the *M. bovis* CF sample. No proteins were detected in CF from the negative control, *M. smegmatis* (cosmid 44).

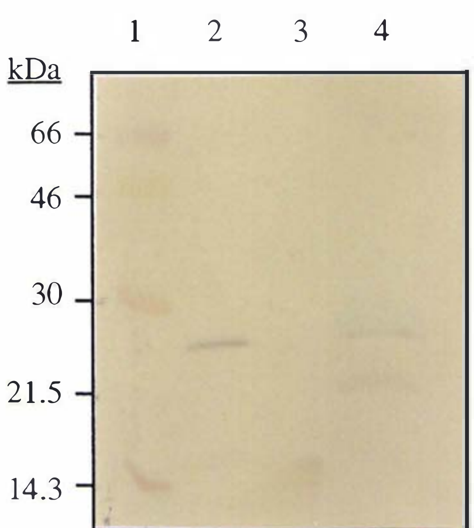



**Figure 4.5**

Western blot identification of MPB64 in CF from recombinant *M. smegmatis*. Culture filtrate samples were separated by SDS-PAGE and transferred to nitrocellulose for probing with rabbit anti-MPB64 antisera. Lane 1, molecular weight markers; lane 2, CF from *M. smegmatis* (cosmid 260); lane 3, CF from *M. smegmatis* (cosmid 44); lane 4, *M. bovis* (KML) CF (positive control).

### 4.3.5 Screening culture filtrates from recombinant *M. smegmatis* for antigens that stimulate T-lymphocyte proliferation

The development of a cell-mediated immune (CMI) response against tuberculosis involves the stimulation of T lymphocytes by mycobacterial antigens which are processed and presented on the surface of antigen-presenting cells. The recognition of antigens can be measured through the activity of antigen-specific T lymphocytes, and monitored *in vitro* by T lymphocyte proliferation and IFN-γ production.

Culture filtrates from the selected *M. smegmatis* recombinants were tested *in vitro* for their ability to stimulate proliferation of PBMCs taken from *M. bovis* BCG-immunised cattle. Peripheral blood mononuclear cells from two control cattle (animals 8 and 23) were also incubated with the CFs to monitor cross reactive T lymphocyte responses. The animals used in the study were selected on the basis of their low T cell reactivity against bovine and avian purified protein derivative (PPD-b and PPD-a, respectively), and *M. smegmatis* CF (Gormley *et al.*, 1996). Four cattle (animals 5, 12, 15 and 21) were immunised subcutaneously on two occasions, 10 weeks apart, with approximately $10^5$ c.f.u. of *M. bovis* BCG (Pasteur). The immune responses of the animals were monitored throughout the study by lymphocyte proliferation and production of IFN-γ in response to *M. bovis* BCG CF, PPD-b and PPD-a (Gormley *et al.*, 1996). Peripheral blood mononuclear cells from animals 21 (immunised) and 23 (control) had consistently high background levels of proliferation (i.e. proliferation in the absence of added antigen). This created a bias in the stimulation indices (SI's) for the recombinant *M. smegmatis* and *M. bovis* BCG CFs, making it difficult to assess the immune responses. The proliferative responses to *M. bovis* BCG CF varied greatly between the immunised animals and the non-immunised control (Table 4.2). Peripheral blood mononuclear cells from the immunised animals (5, 12, and 15) consistently proliferated in response to *M. bovis* BCG CF, with SI's ranging from 12 to 470. In contrast, no proliferation above

background (SI <1.0) was observed when PBMCs taken from the control (animal 8) were incubated with *M. bovis* BCG CF.

Culture filtrates from *M. smegmatis* and *M. smegmatis* (pYUB18) were not used as negative controls as they induced high levels of lymphocyte proliferation compared to CFs from *M. smegmatis* recombinants containing cosmids with DNA inserts (Carpenter and Gormley, pers. comm., 1995). The experiments were internally controlled by comparing responses induced by individual cosmid CF samples.

The selected *M. smegmatis* recombinants were grown in minimal media and CFs prepared. Proliferation assays were initially carried out using three concentrations of the CF proteins, 0.2, 0.5, and 2.0 μg/well. The strongest proliferative response by the PBMCs taken from immunised cattle was at 2.0 μg/well, whereas cells from the control animals remained unresponsive to the CF samples at this concentration. Subsequent experiments were carried out using 2.0 μg CF per well (10 μg/ml).

Differences in the proliferative responses of PBMCs from the immunised animals were observed over time, both in terms of which CF antigens were recognised and the magnitude of the responses (Table 4.2). The proliferation SI's for PBMCs from immunised animals in the presence of the recombinant *M. smegmatis* CF samples peaked at 20 weeks after the booster immunisation. Culture filtrate from *M. smegmatis* (cosmids 196, 260 and 275) induced high levels of proliferation by PBMCs from animal 15 at 25 weeks post boost. These CF samples were not tested at 20 weeks after the booster immunisation. The PBMCs from three immunised animals proliferated strongly in response to CF from *M. smegmatis* (cosmids 44, 48 and 56) compared with the responses to the other recombinant *M. smegmatis* CF samples. In contrast, the proliferative responses to CF from *M. smegmatis* (cosmid 23) were consistently low compared to other CF samples. Culture filtrate from *M. smegmatis* (cosmid 260), which contained the MPB64 protein, also induced low proliferative responses compared to CFs

from other *M. smegmatis* recombinants. The levels of T lymphocyte proliferation induced by CF from recombinant *M. smegmatis* (cosmid 3; containing the *esat-6* gene) were in the middle of the range of responses induced by all recombinant *M. smegmatis* CFs tested.

The proliferation SI's for the recombinant *M. smegmatis* CF samples were higher for immunised animals 5 and 12. Stimulation of PBMCs taken from animal 12 with CF from *M. smegmatis* (cosmid 44) and *M. smegmatis* (cosmid 56) at 20 weeks post boost produced the highest levels of proliferation overall, with SI's of 145.4 and 120.0, respectively (Table 4.2). The proliferative response at this time to *M. bovis* BCG CF was also at its highest (SI = 470) for animal 12.

All of the recombinant *M. smegmatis*, selected on the basis of hybridisation with the pJEM11 recombinants, produced CF that was recognised by PBMCs from immunised cattle. The responses were very variable, however, PBMCs from the control (non-immunised) animal did not proliferate in response to any cosmid CF at any time point.

| Animal | Weeks post boost[a] | Proliferation Stimulation Indices[b] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BCG CF | Recombinant *M. smegmatis* CF samples | | | | | | | | | |
| | | | 2 | 3 | 23 | 44 | 48 | 56 | 68 | 196 | 260 | 275 |
| 5 (i) | 17 | 93.2 | 7.4 | ND | 2.5 | ND | ND | 2.1 | 3.0 | 3.6 | 2.4 | 3.7 |
| | 20 | 82.3 | 46.6 | 29.6 | 7.6 | 44.0 | 43.7 | 36.7 | 30.0 | ND | ND | ND |
| | 25 | 23.3 | ND | 0.4 | ND | 0.6 | 0.6 | ND | ND | 0.6 | 0.9 | 0.5 |
| 12 (i) | 17 | 12.2 | 0.4 | ND | 1.0 | ND | ND | 0.8 | 1.1 | 1.0 | 1.2 | 1.0 |
| | 20 | 470.2 | 12.9 | 39.2 | 3.4 | 145.4 | 45.1 | 120.0 | * | ND | ND | ND |
| | 25 | 18.3 | ND | 0.9 | ND | 1.0 | 0.9 | ND | ND | 1.1 | 0.9 | 1.0 |
| 15 (i) | 17 | 45.2 | 1.5 | ND | 2.0 | ND | ND | 1.2 | 1.5 | * | 1.7 | 1.5 |
| | 20 | 98.2 | 1.4 | * | 4.7 | 6.6 | 19.2 | 15.6 | 4.1 | ND | ND | ND |
| | 25 | 86.1 | ND | 3.6 | ND | 2.0 | 3.3 | ND | ND | 54.3 | 31.0 | 13.0 |
| 8 (c) | 17 | 0.9 | 0.6 | ND | 0.7 | ND | ND | 0.5 | 0.2 | ND | ND | ND |
| | 20 | 0.8 | 0.8 | 0.7 | 0.9 | 0.8 | 0.8 | 0.7 | 0.8 | ND | ND | ND |
| | 25 | 0.6 | ND | 0.2 | ND | 0.2 | 0.1 | ND | ND | 0.4 | 0.1 | 0.3 |

**Table 4.2**

Lymphocyte proliferation responses by PBMCs taken from *M. bovis* BCG-immunised (i), and non-immunised control (c) cattle stimulated with 2.0 µg CF per well. [a] Number of weeks following second immunisation of cattle with *M. bovis* BCG. [b] Results are expressed as stimulation indices (SI's). SI = (mean counts per minute (cpm) of quadruplicate stimulated wells) / (mean cpm of quadruplicate non-stimulated wells). Values outside the median cpm +/- 50% were disregarded in SI calculations. * = all sample values outside the median +/- 50%. ND = not determined.

### 4.3.6 Screening culture filtrates from recombinant *M. smegmatis* for antigens that induce IFN-γ-production by peripheral blood mononuclear cells

Studies in mice suggest that T lymphocyte proliferation alone is not a reliable indicator of a CMI response against mycobacterial infection (Andersen *et al.*, 1992b; Orme *et al.*, 1992). T cell recognition of antigens can also be monitored *in vitro* by IFN-γ production which is considered a more reliable measure of the CMI response.

Culture filtrates from the selected *M. smegmatis* recombinants were tested for their ability to induce IFN-γ-production by PBMCs taken from *M. bovis* BCG-immunised animals. Proliferation assay supernatants were harvested from five day cultures and supernatants from quadruplicate 2.0 μg CF samples were pooled. The pooled supernatants were assayed in duplicate for the presence of IFN-γ, using a sandwich ELISA.

The results of IFN-γ-production by PBMCs from two *M. bovis* BCG-immunised cattle (animals 12 and 15) in response to the ten selected recombinant *M. smegmatis* CF samples are shown in Figure 4.6. As with the proliferative responses, IFN-γ-production by PBMCs from immunised cattle varied over time in response to the recombinant *M. smegmatis* CF samples. The CF from *M. smegmatis* (cosmids 44) and *M. smegmatis* (cosmid 56) induced high levels of IFN-γ-production by PBMCs from both immunised animals. The levels of IFN-γ production were highest in response to CF from recombinant *M. smegmatis* harbouring cosmids 44 and 56 (>10 units/ml). A high level of IFN-γ production (approximately 11 units/ml) was also observed in response to CF from *M. smegmatis* (cosmid 48) with animal 15. In contrast, low levels of IFN-γ (<4 units/ml) were produced in response to CF from recombinant *M. smegmatis* containing cosmids 23 and 275 with PBMCs from *M. bovis* BCG-immunised cattle. Culture filtrate from recombinant *M. smegmatis* harbouring cosmid 260 (shown by Western blot analysis to contain the MPB64 protein) induced low levels of IFN-γ-production (<3.2

units/ml). Moderate levels of IFN-γ (<7.5 units/ml) were produced in response to CF from *M. smegmatis* (cosmid 3). Cosmid 3 has been shown to contain the *esat-6* gene, although it is not known whether the ESAT-6 protein is present in the CF of *M. smegmatis* (cosmid 3).

Peripheral blood mononuclear cells from the two immunised cattle consistently produced IFN-γ in the presence of *M. bovis* BCG CF proteins, ranging between approximately 27 and 157 IFN-γ units/ml. IFN-γ-production by PBMCs taken from a non-immunised control animal was significantly lower, with less than or equal to 7.5 IFN-γ units/ml produced in response to *M. bovis* BCG CF proteins.

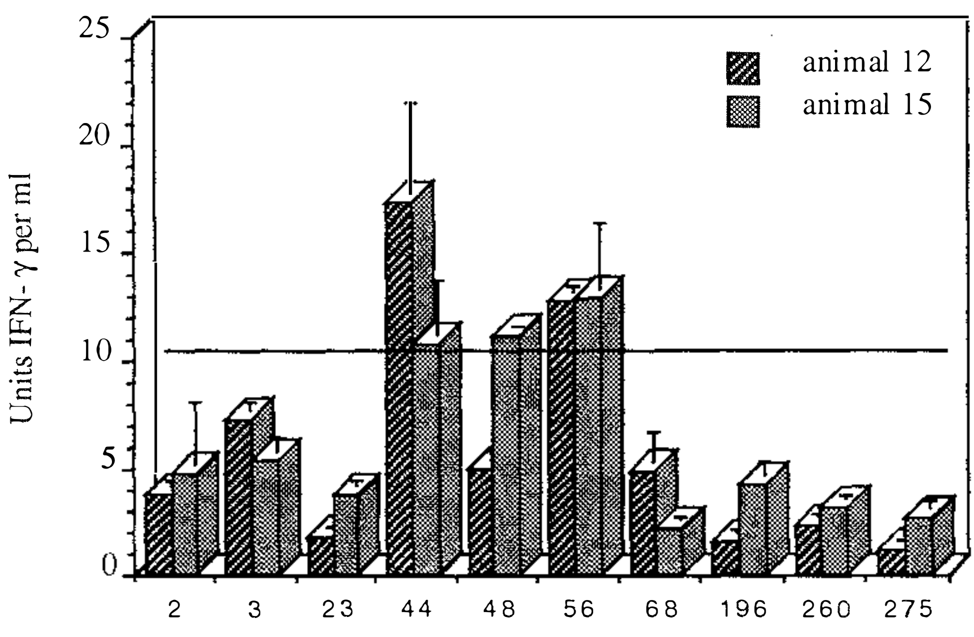



**Figure 4.6**

IFN-γ responses of PBMCs from two *M. bovis* BCG-immunised cattle to ten recombinant *M. smegmatis* CF preparations. Peripheral blood mononuclear cells ($2x10^5$) were cultured with 2.0 μg of CF protein prepared from the ten recombinants. The presence of IFN-γ was determined in supernatants from five day cultures, using a sandwich ELISA. Samples were assayed in duplicate and IFN-γ levels were determined by comparison with a recombinant bovine IFN-γ standard curve. Results, expressed as the mean units of IFN-γ per ml, are representative of two experiments. Error bars represent one standard deviation (stdev) from the mean. The units IFN-γ per ml for each sample and animal were averaged and a line drawn across the graph at the mean plus one stdev. The PBMCs produced IFN-γ up to 157 units/ml in response to *M. bovis* BCG CF (data not shown).

## 4.4 Discussion

An *M. bovis* cosmid library expressed in *M. smegmatis* was screened for the presence of genes coding for exported proteins. Western blot analysis revealed that *M. bovis* proteins could be synthesised and secreted into the medium from *M. smegmatis.* Culture filtrates from *M. smegmatis* recombinants containing *M. bovis* genes coding for exported proteins were screened for the presence of T cell antigens, as measured by their ability to induce proliferation and IFN-γ-production by PBMCs taken from *M. bovis* BCG-immunised cattle.

PCR amplification was used to identify cosmids containing the genes coding for the previously characterised secreted antigens ESAT-6, MPB64, MPB70, 85-B and PhoS. The *esat-6* and *mpb64* genes were mapped to cosmids 3 and 260 respectively. These cosmids were also identified by screening the library with insert DNA from PhoA+ clones. This provided evidence that more than one exported protein gene could be present on a single cosmid insert, and thus questioned the contribution of single or multiple proteins to observed immune responses. To determine if known *M. bovis* secreted antigens were expressed and secreted by their *M. smegmatis* hosts, CF from *M. smegmatis* (cosmid 260) was subjected to Western blot analysis. A 26 kDa protein band was detected by the anti-MPB64 polyclonal antibodies in the *M. smegmatis* (cosmid 260) and *M. bovis* CF samples. This is similar to the molecular weight of recombinant MPB64 / MPT64 produced by recombinant *M. smegmatis* in other studies (Tasaka *et al.*, 1995; Roche *et al.*, 1996). In these studies, the size and tertiary conformation of recombinant MPT64/MPB64 were the same as the native form of the protein. Western blot analysis has also revealed that recombinant 85-B antigen is expressed and secreted by *M. smegmatis* (cosmid 79) and is the same molecular weight as the native *M. bovis* BCG protein (Gormley *et al.*, 1996). The results of the Western blot experiments carried out in this study, indicate that the cosmid-encoded *M. bovis* secreted proteins are not degraded by their *M. smegmatis* host, in contrast to the PhoA fusion proteins examined

in Chapter 3. This provided evidence that stably formed proteins were more resistant to degradation by host proteases compared to fusion polypeptides.

Short-term CFs of *M. tuberculosis* and *M. bovis* are major targets of T cell responses to these mycobacteria. The MPT64 protein is present in *M. tuberculosis* CFs, and is recognised by tuberculosis patients but not *M. bovis* BCG vaccinated humans (Roche *et al.*, 1994). The MPB64 protein is also a major target for immune recognition in *M. bovis*-infected cattle (Fifis *et al.*, 1994). The *M. bovis* BCG (Pasteur) strain used to immunise the animals in this study, has previously been shown to lack the *mpb64* gene (Li *et al.*, 1993). Therefore any immune responses to CFs from *M. smegmatis* (cosmid 260) would presumably be directed against other protein components of the CF. In spite of the observation that recombinant MPB64 was secreted in sufficient quantities to be detected by antibodies, the levels of proliferation and IFN-γ-production by PBMCs taken from immunised animals were low compared to other recombinant *M. smegmatis* CF samples tested. Similar results were obtained with CF from recombinant *M. smegmatis* (cosmid 3; containing the *esat-6* gene). The *esat-6* gene is also known to be deleted from *M. bovis* BCG (Harboe *et al.*, 1996). These results suggest that CF prepared from *M. smegmatis* (cosmid 3) and *M. smegmatis* (cosmid 260) do not contain antigens which induce strong immune responses in these immunised animals.

The CFs prepared from the recombinant *M. smegmatis* selected by hybridisation to insert DNA from PhoA+ clones were screened for their ability to induce proliferation and IFN-γ-production by PBMCs taken from *M. bovis* BCG-immunised and non-immunised control cattle. Culture filtrates from *M. smegmatis* (cosmid 44) and *M. smegmatis* (cosmid 56) induced IFN-γ-production by PBMCs from two *M. bovis* BCG-immunised animals. The CF from these *M. smegmatis* recombinants were also able to induce proliferation of lymphocytes from all *M. bovis* BCG-immunised cattle tested. It is likely, therefore, that CF preparations from *M. smegmatis* (cosmid 44) and *M. smegmatis* (cosmid 56) contain one or more cosmid-encoded T cell antigens. Protein analysis by

SDS-PAGE did not reveal any visible differences between the *M. smegmatis* recombinants, indicating that the secreted recombinant proteins represent a small proportion of the total CF protein.

Variation was observed in the immune responses of the immunised animals to the individual recombinant *M. smegmatis* CF samples. This heterogeneity of responses was not unexpected in relatively outbred animals such as cattle, and has previously been observed in *M. bovis* BCG-immunised animals (Carpenter and Gormley, pers. comm., 1995) and *M. bovis*-infected cattle (Fifis *et al.*, 1994). In the Fifis study using *M. bovis*-infected cattle, there was also considerable variation in the responses to purified *M. bovis* antigens over time, with different antigens being recognised at different stages of the infection.

In general, the magnitude of the T cell proliferative responses induced by each CF preparation correlated with the IFN-γ responses. Culture filtrates from *M. smegmatis* (cosmid 44) and *M. smegmatis* (cosmid 56) induced the highest responses in both the proliferation and IFN-γ assays, whereas CF from *M. smegmatis* (cosmid 23) induced minimal responses in each assay. In a study using *M. bovis* infected cattle, IFN-γ responses have been shown to be highly reproducible (Rothel *et al.*, 1992). While the results of the IFN-γ assays in the present study showed some variability between animals and over time, they were more consistent than the lymphocyte proliferation assays. This suggests that IFN-γ-production by PBMCs from *M. bovis* BCG-immunised animals is a more reliable measure of T cell reactivity than lymphocyte proliferation.

The observation that recombinant *M. smegmatis* CF samples (cosmids 44 and 56) induced the highest responses in both the proliferation and IFN-γ assays demonstrated the usefulness of the *M. smegmatis* expression system for the detection of T lymphocyte-reactive antigens. Although the recombinant *M. smegmatis* CFs contained a complex mixture of proteins, the screening system could distinguish between *M. smegmatis*

proteins and *M. bovis* antigens. This was also true when the magnitude of individual animal's responses varied. This might have been expected as the immunised animals used in this study were chosen on the basis of low lymphocyte reactivity against *M. smegmatis* CF proteins. Following immunisation and routine testing of CMI responses, the cross-reactive responses to *M. smegmatis* and *M. smegmatis* (pYUB18) were found to be considerably greater than responses to randomly selected *M. smegmatis* containing pYUB18 with *M. bovis* DNA inserts (Carpenter *et al.*, 1995). It is possible that the increased genetic load on the host *M. smegmatis* containing large inserts could alter the synthesis and secretion patterns of host native proteins.

Many of the previously characterised mycobacterial antigens were originally detected by screening with antisera taken from tuberculin positive patients (Amara and Satchidanandam, 1996), or with monoclonal antibodies generated against mycobacterial proteins (Young *et al.*, 1985). More direct approaches involving biochemical fractionation of CF proteins and assaying directly for T cell activity have resulted in the detection of several immunodominant antigens. Recombinant cosmid libraries, as described in this study, offer an alternative, convenient method of antigen detection. The large insert sizes provide an advantage over conventional plasmid or phage libraries in that relatively few clones are necessary for full representation of a complete genome. When the library is cloned in a heterologous, but related host, the probability is increased that the gene expression signals will be recognised. In addition, protein export signals and signals involved in post-translational modifications may also function in the alternative host. The identification of secreted antigens in CFs prepared from recombinant *M. smegmatis* demonstrates the potential of this heterologous expression system for the analysis of a wide range of *M. bovis* secreted antigens.

# Chapter 5:

# Characterisation Of An *M. bovis* Putative Exported Lipoprotein (Pel) With Homology To A Family Of Maltose-Binding Proteins

## 5.1 Abstract

This chapter describes the characterisation of an *M. bovis* putative exported lipoprotein (Pel). The *pel* gene was subcloned from cosmid 56 of the *M. bovis* cosmid library, and its nucleotide sequence determined and analysed. Database searches with the predicted amino acid sequence of *M. bovis* Pel revealed similarity to an *M. leprae* putative lipoprotein and a family of maltose-binding proteins. A search of adjacent genes in *M. leprae* revealed two ORFs, coding for putative maltose transport proteins, located downstream of the *pel* homologue. The start of an ORF, with similarity to a putative *M. leprae* maltose transport protein, was identified downstream of the *M. bovis pel* gene. Analysis of loci regulating maltose and maltodextrin transport in other bacteria suggested that the *M. bovis pel* gene and its *M. leprae* homologue may be part of an operon involved in maltose and maltodextrin transport. Southern blot analysis indicated that both slow and fast-growing mycobacterial species contain sequences related to the *pel* gene. The *M. bovis pel* gene was expressed by recombinant *M. smegmatis* as shown by Northern blot analysis.

*M. smegmatis* recombinants containing the *M. bovis pel* gene and a randomly selected, cloned *M. bovis* DNA fragment were grown in culture and the supernatants collected. The CFs were tested *in vitro* for their ability to induce proliferation and IFN-γ-production by PBMCs taken from *M. bovis* BCG-immunised and non-immunised, control cattle. The level of IFN-γ-production by PBMCs from one of the *M. bovis* BCG-immunised animals was two-fold higher in the presence of CF from the recombinant expressing the *pel* gene.

## 5.2 Results

### 5.2.1 Subcloning of the *M. bovis* putative exported lipoprotein gene from cosmid 56

The pSUM family of *Mycobacterium* / *E. coli* shuttle vectors contain seven unique cloning sites, a kanamycin resistance gene, and a *lacZα* reporter gene for screening for recombinants in *E. coli* (Ainsa *et al.*, 1996). The universal M13 sequencing primers can be used to obtain insert DNA sequences adjacent to the multiple cloning site (MCS).

The plasmid construct pM21 contains a 1.5 kb *M. bovis* BCG DNA insert cloned into the pJEM11 vector (Chapter 3). DNA sequence analysis showed that the pM21 plasmid contains a partial putative exported lipoprotein (*pel*) gene fused to *phoA*. Southern blot analysis revealed that the *pel* gene is located on cosmid 56 of the *M. bovis* cosmid library. A restriction map of the pM21 insert DNA was generated to identify potentially suitable enzymes for subcloning the full length gene from cosmid 56. Five restriction enzymes (*Bam* HI, *Cla* I, *Hin* dIII, *Sph* I and *Xba* I) which did not cut within the pM21 ORF or its immediate upstream region were compatible with sites in the MCS of the pSUM vectors. Cosmid 56 DNA was purified from recombinant *E. coli* and digested with these five restriction enzymes. A randomly selected cosmid was also digested to serve as a negative control. Following electrophoresis, the DNA fragments were blotted onto nylon membranes and probed with radiolabelled insert DNA from pM21. The probe hybridised to a single DNA fragment in each digested cosmid 56 sample. The smallest fragment to which the probe hybridised, a *Cla* I-fragment of approximately 8.3 kilobases (kb), was selected for subcloning.

DNA fragments generated by *Cla* I-digestion of cosmid 56 were separated by electrophoresis. Two fragments, of approximately 8.3 and 10 kb, were excised together and purified from the agarose gel. The *Cla* I-fragments were ligated into *Cla* I-digested,

dephosphorylated pSUM40, and used to transform *E. coli*. The two cosmid subclones (subclones 56/1 and 56/2) were isolated and purified from recombinant *E. coli*. Southern blot analysis of the subclones 56/1 and 56/2 was carried out to determine which subclone contained the exported lipoprotein gene (Figure 5.1). The pM21 insert DNA probe hybridised to the DNA insert of subclone 56/2 (approximately 8.3 kb). A fragment of identical size in the cosmid 56 control sample, and a larger fragment (>10 kb) in the *Cla* I-digested *M. bovis* genomic DNA control also hybridised to the DNA probe. No bands were observed in the lanes containing the digests of subclone 56/1 or the randomly selected cosmid (negative control). This indicated that subclone 56/2 contained the *M. bovis* equivalent sequence of at least part of the *M. bovis* BCG DNA sequence of the pM21 insert.

To determine if the full length *pel* gene was present in subclone 56/2, the universal M13 sequencing primers were used to sequence the ends of the DNA insert. Database searches using sequence data generated with the M13 reverse primer revealed similarity to sequences in an *M. leprae* cosmid (B1756) that contained an homologous *pel* gene. These sequences mapped approximately 5.65 kb downstream from the 3' end of the *pel* gene. Sequence obtained with the M13 forward primer showed similarity to the pAL5000 sequence from *M. fortuitum*, suggesting that part of the pYUB18 cosmid vector was contained within the ~8.3 kb *Cla* I-fragment insert of subclone 56/2. This explained the size differences between the observed *M. bovis* genomic DNA *Cla* I-fragment, and the cosmid 56 and subclone 56/2 fragments (Figure 5.1). DNA sequencing of subclone 56/2, using a primer designed to hybridise approximately 600 nucleotides upstream of the pM21 ORF (*pel-phoA*), confirmed that *M. bovis* DNA sequences upstream of the *pel* gene were identical to the *M. bovis* BCG sequences of the pM21 insert.

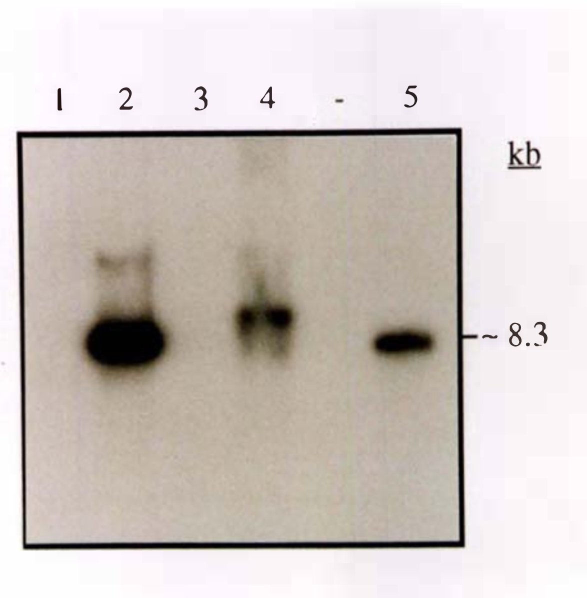



**Figure 5.1**

Southern blot of the *M. bovis* cosmid 56 subclones probed with insert DNA from pM21. DNA samples were digested with *Cla* I, electrophoresed, and blotted onto nylon membranes for probing with the pM21 DNA insert. Lane 1, subclone 56/1; lane 2, subclone 56/2; lane 3, cosmid 51 (negative control); lane 4, *M. bovis* (KML) genomic DNA; and lane 5, cosmid 56.

### 5.2.2 Nucleotide sequence of the *M. bovis pel* gene

The complete nucleotide sequence of the *pel* gene located in subclone 56/2 was determined on both strands using PCR cycle sequencing. Oligonucleotide primers designed to sequence the *M. bovis* BCG pM21 insert (Appendix 2B) were used to sequence the corresponding region of the *M. bovis* DNA insert of subclone 56/2. The *M. bovis* and *M. bovis* BCG sequences were identical over the 1254 nucleotide bases common to the pM21 and subclone 56/2 DNA inserts. Additional oligonucleotide primers were generated based on the available sequence data (Appendix 2C), and the full length gene was sequenced in a stepwise manner using subclone 56/2 DNA as a template for PCR cycle-sequencing.

The nucleotide and deduced amino acid sequences of the *M. bovis* Pel protein are shown in Figure 5.2. The *M. bovis* 1404 bp ORF extends from a putative GTG start codon at position 361 to a TGA stop codon at position 1825. A potential Shine-Dalgarno (SD) motif (GAGAGG) is located at position -6 with respect to the putative GTG start codon. A second potential start codon (ATG) is found at position 367, 12 bases downstream from the potential SD sequence. The G+C content of the ORF (66.1%) is consistent with the global G+C content observed in the mycobacterial genome.

The *M. bovis* ORF potentially codes for a 468 amino acid polypeptide with a putative lipoprotein signal peptide of 25 amino acids. The sequence adjacent to the putative cleavage site (AAA↓C) is consistent with the lipoprotein signal peptidase consensus (L,V,I)(A,S,T,G)(G,A) ↓C (von Heijne, 1989). The calculated molecular mass of the polypeptide precursor is 49.8 kDa, and the mature Pel protein is 47.3 kDa. This is of similar size to the homologous *M. leprae* 1407 bp ORF, which encodes a 469 amino acid polypeptide precursor with a 26 amino acid putative signal peptide. The *M. leprae* Pel precursor contains a sequence (VLA↓C) similar to the lipoprotein signal peptidase consensus. The *M. leprae* Pel homologue polypeptide precursor has a calculated

molecular mass of 50.5 kDa, and the mature protein is 47.8 kDa. Alignment of the *M. bovis* and *M. leprae* ORFs indicates that they are 76% identical at the nucleotide level. The deduced amino acid sequences are 78% identical and 88% similar between species.

The amino acid composition of the full-length *M. bovis* Pel protein reveals a high percentage (16%) of alanine residues. The next most abundant amino acid is leucine at 9.8%, followed by valine, threonine, proline and glycine, which each make up 7.1 to 7.5% of the protein. These amino acids contain non-polar side chains, with the exception of threonine which has an uncharged, polar side chain. A hydrophobicity plot, based on the Kyte-Doolittle predictions is shown in Figure 5.3. The N-terminal putative signal peptide is the most hydrophobic region of the protein, and is followed by a relatively hydrophilic region of approximately 175 amino acid residues. The middle section of the primary sequence (amino acids 200 to 360) is predominantly hydrophobic, although three weakly hydrophilic domains are evident. The remaining C-terminal protein sequence is predominantly hydrophilic with the exception of a hydrophobic stretch of approximately 20 residues.

The predicted isoelectric point (pI) of the mature 47 kDa Pel protein is 4.94. Two-dimensional gel electrophoresis of CF from *M. bovis* BCG has revealed that the majority of secreted proteins have a pI values between 4.5 and 6.0 (Gulle *et al.*, 1995). A cluster of proteins has been observed between 45 and 50 kDa at approximately pH 4.9.

**Figure 5.2**

Nucleotide sequence of the gene encoding the *M. bovis* Pel protein. The nucleotide sequence contains an ORF of 1404 base pairs. The putative initiation codon (GTG) is preceded by a potential Shine-Dalgarno sequence (GAGAGG) 6 bp upstream. The deduced 468 amino acid sequence is shown below. The putative cleavage site of the lipoprotein signal peptide is marked with an arrow, suggesting that the Pel protein has a 26 amino acid signal peptide. Nucleotides in italics were determined by sequencing on a single DNA strand.

```
GACCATCGTTGGCGTGGACCTCATGCAGGTTGAACGGGTCACAGGCCGGCTGACCTGGCC     60

CAAAGTGCTTGGTGGCGGCGTGCTGAGTGGCGCCTGGCTGGGCCTGTTCATCGGGTTGGT    120

GCTCGGGTTCTTCAGTCCCAATCCATGGTCCGCGCTGGTTACCGGCCTGGTGGCCGGGGT    180

GTTCTTCGGGCTGATCACCTCTGCAGTGCCGTACGCAATGGCTCGCGGCACAAGGGATTT    240

CAGCTCGACCATGCAACTGGTTGCCGGTCGCTACGACGTACTTTGTGATCCGCAAAATGC    300

GGAAAAGGCACGGGATCTGCTGGCGCGTCTGGCGATCTGAAGCCCGGACGAGAGGCAAAT    360

GTGGTCATGAGTCGCGGGCGGATACCGAGGCTGGGCGCTGCCGTACTGGTGGCGTTGACG    420
 M  V  M  S  R  G  R  I  P  R  L  G  A  A  V  L  V  A  L  T

ACCGCGGCGGCGGCGTGCGGGGCCGATAGCCAGGGGCTGGTGGTCAGCTTCTACACACCG    480
 T  A  A  A  A  C ↑G  A  D  S  Q  G  L  V  V  S  F  Y  T  P

GCCACCGACGGCGCGACGTTCACCGCAATTGCCCAACGCTGCAACCAACAGTTCGGCGGC    540
 A  T  D  G  A  T  F  T  A  I  A  Q  R  C  N  Q  Q  F  G  G

CGGTTCACCATTGCGCAGGTCAGCTTGCCCAGGTCCCCCAATGAGCAACGGTTACAGCTG    600
 R  F  T  I  A  Q  V  S  L  P  R  S  P  N  E  Q  R  L  Q  L

GCCCGACGGTTGACCGGTAACGACCGCACCCTGGACGTCATGGCGCTGGATGTGGTGTGG    660
 A  R  R  L  T  G  N  D  R  T  L  D  V  M  A  L  D  V  V  W

ACGGCGGAGTTCGCCGAAGCGGGGTGGGCGCTGCCGCTGTCGGACGACCCAGCGGGGCTG    720
 T  A  E  F  A  E  A  G  W  A  L  P  L  S  D  D  P  A  G  L

GCCGAGAACGACGCCGTCGCCGATACCCTGCCAGGCCCGCTTGCGACGGCCGGCTGGAAC    780
 A  E  N  D  A  V  A  D  T  L  P  G  P  L  A  T  A  G  W  N

CACAAGCTGTACGCGGCACCCGTCACCACTAATACTCAATTGCTTTGGTACCGACCAGAT    840
 H  K  L  Y  A  A  P  V  T  T  N  T  Q  L  L  W  Y  R  P  D
```

TTGGTAAATAGCCCGCCAACGGATTGGAATGCCATGATCGCTGAGGCGGCCCGGCTGCAC 900
L V N S P P T D W N A M I A E A A R L H

GCGGCGGGCGAGCCTAGCTGGATCGCGGTACAGGCCAATCAGGGCGAGGGCTTAGTGGTG 960
A A G E P S W I A V Q A N Q G E G L V V

TGGTTCAACACGCTGCTGGTGAGCGCTGGTGGATCGGTGCTCTCCGAGGACGGCCGGCAC 1020
W F N T L L V S A G G S V L S E D G R H

GTCACCTTGACCGATACTCCCGCACACCGAGCGGCTACGGTCAGCGCGCTACAGATCCTC 1080
V T L T D T P A H R A A T V S A L Q I L

AAATCGGTGGCTACCACGCCCGGCGCCGACCCCTCGATCACCCGCACCGAAGAGGGCAGC 1140
K S V A T T P G A D P S I T R T E E G S

GCGCGGTTGGCCTTCGAACAGGGCAAGGCCGCGCTCGAGGTCAATTGGCCGTTCGTGTTT 1200
A R L A F E Q G K A A L E V N W P F V F

GCGTCCATGCTCGAGAACGCGGTGAAGGGTGGTGTGCCCTTCTTACCGCTTAACCGGATT 1260
A S M L E N A V K G G V P F L P L N R I

CCGCAGTTGGCCGGCAGCATCAACGACATCGGGACGTTCACGCCCAGCGACGAGCAGTTC 1380
P Q L A G S I N D I G T F T P S D E Q F

CGCATCGCGTATGACGCCAGCCAGCAGGTGTTCGGTTTCGCGCCCTATCCGGCTGTAGCG 1440
R I A Y D A S Q Q V F G F A P Y P A V A

CCCGGCCAGCCAGCCAAGGTGACGATCGGCGGGTTGAACCTGGCGGTGGCCAAGACGACC 1500
P G Q P A K V T I G G L N L A V A K T T

CGCCATCGAGCGGAGGCATTCGAAGCGGTGCGTTGTCTGCGTGACCAGCACAATCAGAGG 1560
R H R A E A F E A V R C L R D Q H N Q R

TACGTCTCGCTCGAGGGGGGTCTGCCCGCGGTGCGGGCGTCGCTGTACTCCGATCCGCAA 1620
Y V S L E G G L P A V R A S L Y S D P Q

TTCCAGGCGAAGTATCCGATGCACGCCATTATTCGGCAGCAACTCACCGATGCCGCGGTG 1680
F Q A K Y P M H A I I R Q Q L T D A A V

CGGCCGGCGACGCCGGTGTACCAGGCGTTGTCCATCCGGCTCGCGGCGGTGCTGAGCCCG 1740
R P A T P V Y Q A L S I R L A A V L S P

ATCACCGAGATCGACCCGGAGTCCACGGCCGACGAACTTGCCGCGCAGGCGCAGAAAGCC 1800
I T E I D P E S T A D E L A A Q A Q K A

ATCGACGGCATGGGCCTGCTCCCGTGACCTCCGTTGAACAGCGGACCGCCACCGCGGT*CT* 1860
I D G M G L L P *

*TTTCCCGTACCGGGAGCCGCATGGCCGAACGGCGACTGGCGTTCATGCTGGTCGCACCCG* 1920

*CCGCGATGTTGATGGTGGCGGTGACGGCCTATCCCATCGGTTA* 1963

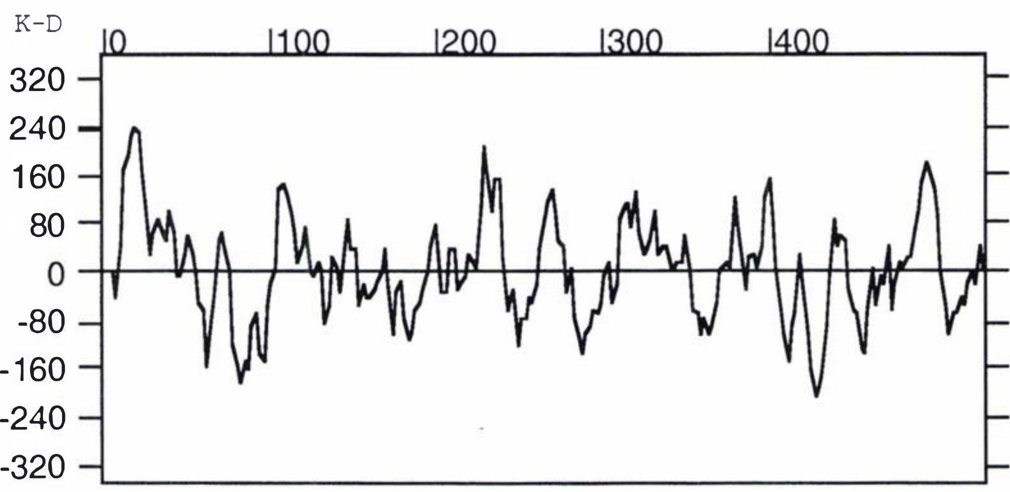



**Figure 5.3**

Hydropathy analysis (Kyte-Doolittle) plot of the deduced amino acid sequence of the *M. bovis* Pel protein. Plots above the line indicate a hydrophobic nature. The numbers on the horizontal axis represent amino acid numbers within the polypeptide sequence. The hydrophobic index (y-axis) was calculated using an interval of 11 amino acids and multiplying hydrophobicity values by ten.

### 5.2.3 Similarity between the *M. bovis* Pel protein and a family of maltose-binding proteins.

Databases were searched with the *M. bovis* Pel predicted amino acid sequence using the BLAST (Altschul *et al.*, 1990) and BEAUTY (Worley *et al.*, 1995) algorithms to locate proteins, other than the *M. leprae* Pel homologue, with similarity to the *M. bovis* Pel protein. The search results revealed two regions of similarity to the maltose-binding protein (MalE) precursor of the Gram negative bacterium *Enterobacter aerogenes* (47% over 46 amino acids, and 60% over 41 amino acids; Figure 5.4) (Dahl *et al.*, 1989). The database search also identified lesser similarity to MalE maltose-binding proteins from Gram negative *E. coli*, *Salmonella typhimurium*, and *Thermotoga maritima*, and Gram positive *Streptomyces coelicolor*, and *Alicyclobacillus acidocaldarius*. These MalE proteins form part of a multi-component maltose-transport system.

The homologous regions of the *M. bovis* Pel protein and *Enterobacter aerogenes* MalE precursor are shown in Figure 5.4. The BLAST search results indicated that the probability of these amino acid similarities occurring randomly is $6.2 \times 10^{-5}$. The regions of similarity spanned three of the eight maltose-binding protein motifs present in the *Enterobacter aerogenes* protein sequence (data not shown). The *Enterobacter aerogenes* and *M. bovis* protein sequences were aligned using the Geneworks programme, and spaces were introduced to maximise the alignment. Overall, the *M. bovis* Pel protein was 19% identical (90 identical amino acids) and 29% similar (47 conservative substitutions) to the *Enterobacter aerogenes* MalE precursor (data not shown).

```
M. bovis       139 WNHKLYAAPVTTNTQLLWYRPDLVNSPPTDWNAMIAEAARLHAAGE 184
                   +N.KL.A.PV......L.Y..DLV.+PP..W..+.A....L.A.G+
E. aerogenes   125 YNGKLIAYPVAVEALSLIYNKDLVPNPPKTWEEIPALDKALKAKGK 170

                   Identities = 18/46 (39%), Positives = 22/46 (47%)


M. bovis       256 TEEGSARLAFEQGKAALEVNWPFVFASMLENAVKGGVPFLP 296
                   T+...A..AF.+G+.A+..+N.P+.++++ ++.V..GV..LP
E. aerogenes   234 TDYSIAEAAFNKGETAMTINGPWAWSNIDKSKVNYGVTLLP 274

                   Identities = 13/41 (31%), Positives = 25/41 (60%)
```

**Figure 5.4**

Regions of sequence similarity between the *M. bovis* putative exported lipoprotein (469 amino acids) and the *Enterobacter aerogenes* maltose-binding protein precursor (396 amino acids). Amino acid sequences are shown using the single letter code. Regions of similarity were identified using the BLAST and BEAUTY algorithms. The probability of these sequences aligning purely by chance is $6.2 \times 10^{-5}$.

### 5.2.4 The *pel* genes of *M. leprae* and *M. bovis* may be part of an operon involved in maltose transport.

When the *M. bovis* Pel protein sequence was used in database searches, the coding region of the homologous *M. leprae* Pel sequence mapped upstream of two ORFs, coding for the putative maltose transport inner membrane proteins MalF and MalG. Hydropathy analysis of *M. leprae* MalF and MalG proteins revealed several hydrophobic domains in each protein (Figure 5.5), indicating that these proteins might be transmembrane proteins. In contrast, hydropathy plots of the *M. bovis* and *M. leprae* Pel proteins revealed considerably fewer and shorter hydrophobic domains (Figure 5.5).

The nucleotide sequence downstream of the *M. bovis pel* gene was analysed for ORFs with homology to the *M. leprae malF* gene. The start of an ORF was identified 53 bases downstream from the putative TGA stop codon of the *M. bovis pel* gene. An alignment of the 27 amino acid product of this partial ORF and the *M. leprae* MalF protein is shown in Figure 5.6. The amino acid sequence of the *M. bovis* putative MalF protein was 74% identical and 85% similar to the aligned *M. leprae* MalF sequence. The probability of this degree of similarity between the *M. leprae* and *M. bovis* protein sequences occurring by chance is $1.6 \times 10^{-8}$ (Altschul *et al.*, 1990).

The transport of maltose and maltodextrin in *E. coli* is regulated by the *malB* locus (Hofnung, 1974). The *malB* locus consists of two divergently transcribed operons, *malEFG* and *malKM-lamB*. From the database searches, MalE and MalF appear to have homologues in *M. bovis* and *M. leprae*, as well as a MalG homologue in *M. leprae*. The *E. coli* MalK, MalM and LamB protein sequences were used in BLAST searches to locate homologous mycobacterial proteins. The search with MalK revealed similarity to an *M. leprae* putative UgpC protein. The *M. leprae ugpC* gene (31,796-32,974) mapped immediately downstream from *malG* (30,901-31,791) in cosmid B1756. *M. leprae* UgpC showed 57% identity and 76% similarity to *E. coli* MalK over 198 amino acids.

The probability of this similarity occurring purely by chance is $6.0 \times 10^{-85}$. The relative map positions of the *pel* , *malF*, *malG*, and *ugpC* genes within the *M. leprae* genome are shown in Figure 5.7.

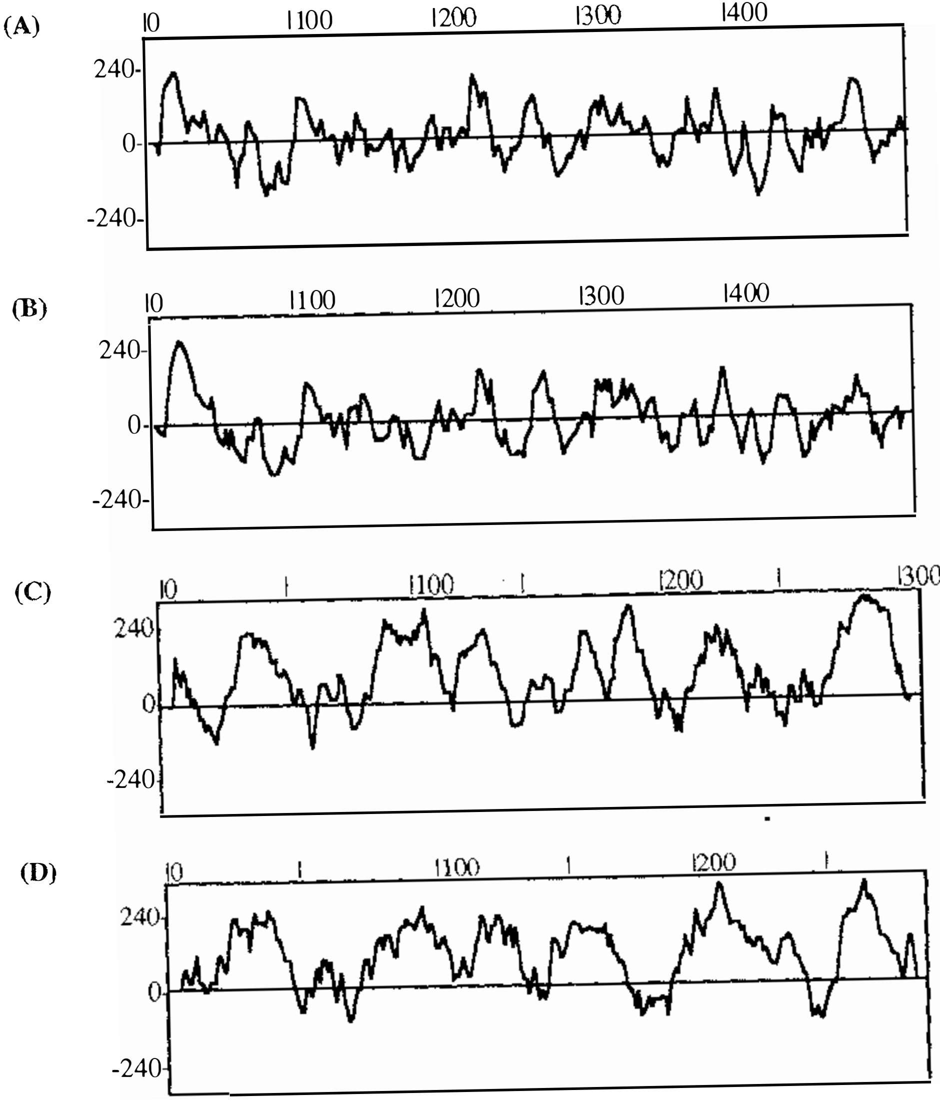



**Figure 5.5**

Hydropathy analysis (Kyte-Doolittle) plots of (A) the *M. bovis* Pel protein, and the *M. leprae* (B) Pel, (C) MalF, and (D) MalG proteins. Hydrophobic domains are above the horizontal line. The numbers on the x-axis represent the amino acid number of each protein.

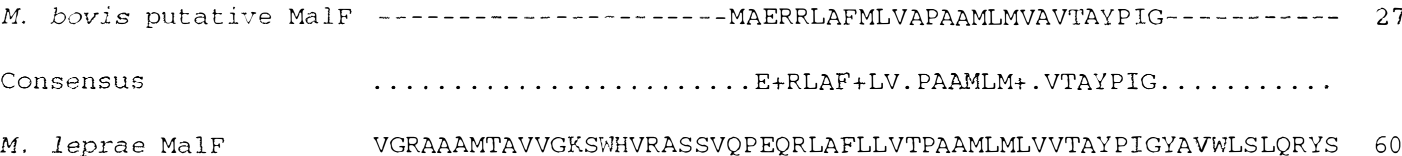



**Figure 5.6**

Region of sequence similarity between the product of an ORF downstream of the *M. bovis pel* gene and the *M. leprae* MalF protein. Amino acid sequences are shown using the single letter code. The amino acid sequences of the *M. leprae* and *M. bovis* proteins are 74% identical (20/27 residues) and 85% similar (23/27 residues).

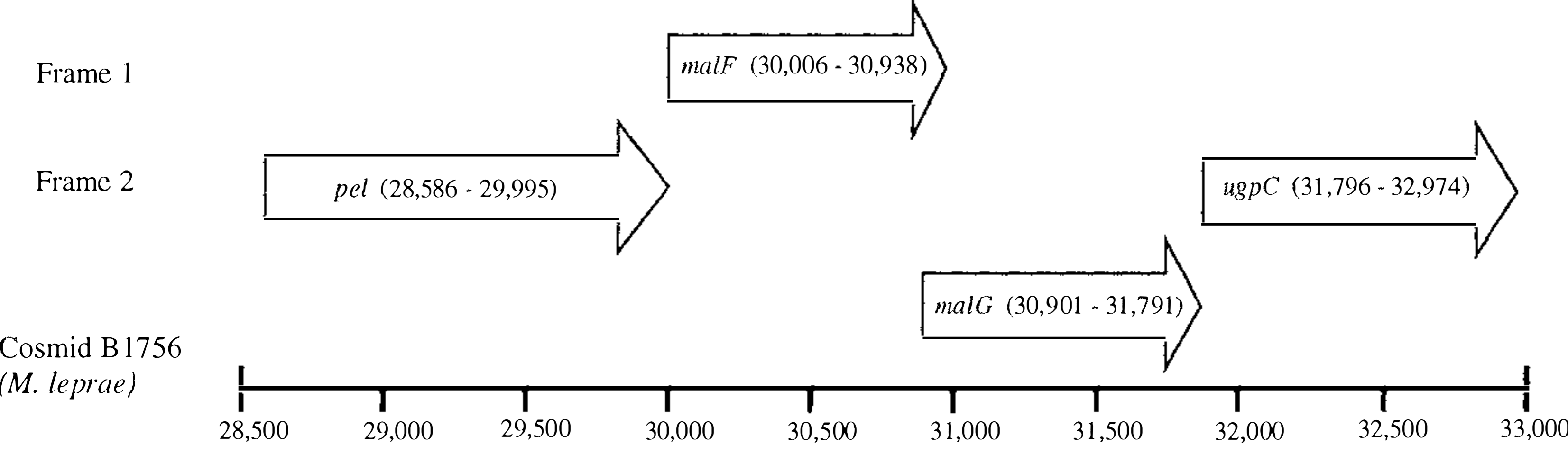



**Figure 5.7**

Genomic arrangement of a putative operon involved in maltose transport in the *M. leprae* cosmid B1756. Database searches with the *M. bovis pel* gene identified an homologous *pel* gene in *M. leprae*. Three ORFs were located downstream of the *M. leprae pel* homologue, named *malF, malG* and *ugpC*. The *pel* and *ugpC* gene sequences show strong similarity to *malE* and *malK* genes, respectively. The numbers along the horizontal axis represent the nucleotide number within cosmid B1756.

### 5.2.5 Species distribution of the *M. bovis pel* gene

To determine if mycobacteria, other than *M. leprae,* contained gene sequences related to *pel*, 15 species of mycobacteria were examined by Southern blot analysis. Genomic DNA were prepared from the mycobacteria and *Eco* RI-digested DNA fragments were transferred to nylon membranes following electrophoresis. The membrane was probed, using high stringency hybridisation conditions, with a 234 bp fragment of the *pel* gene, generated by digestion of pM21 with *Kpn* I and *Pvu* II. Exposure of the Southern blot membrane to X-ray film for seven days revealed that all the mycobacterial species tested contained sequences which hybridised to the partial *pel* gene probe (Figure 5.8). The probe hybridised strongly to DNA fragments of identical size (approximately 6.0 kb) in the *M. bovis*, *M. bovis* BCG, and *M. tuberculosis* samples. *M. avium*, *M. intracellulare* and *M. paratuberculosis* DNA fragments of approximately 7 kb hybridised weakly to the partial *pel* gene probe. The probe hybridised weakly to DNA fragments of greater than or equal to 15 kb in the remaining mycobacterial samples.

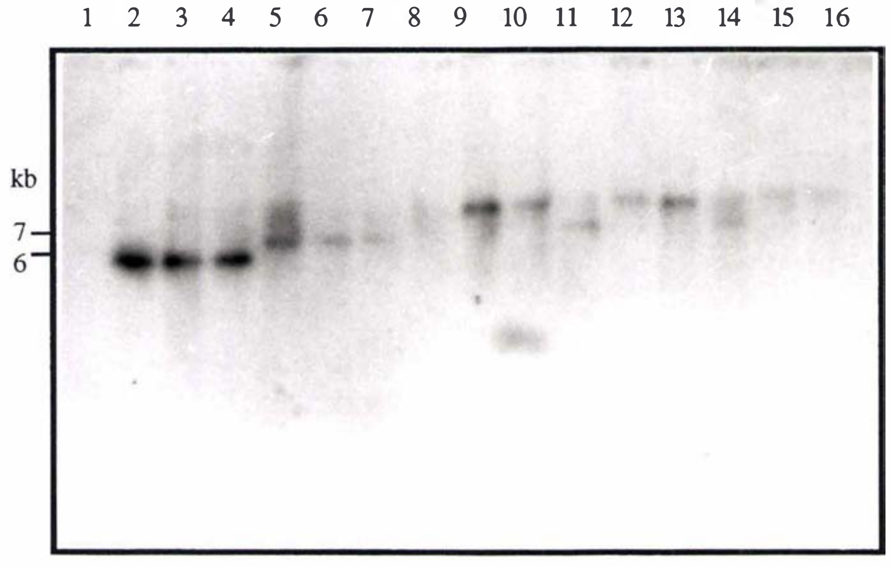



**Figure 5.8**

Southern blot of *Eco* RI-digested mycobacterial genomic DNA probed with a 234 bp fragment from the *M. bovis* BCG insert of pM21 corresponding to part of the *pel* gene. Lane 1, DNA size standards (1 kb extension ladder). Lanes 2 to 16 contain DNA digests from the following species; (2) *M. tuberculosis,* (3) *M. bovis,* (4) *M. bovis* BCG, (5) *M. avium,* (6) *M. intracellulare,* (7) *M. paratuberculosis,* (8) *M. kansasii,* (9) *M. scrofulaceum,* (10) *M. gordonnae,* (11) *M. marinum,* (12) *M. terrae,* (13) *M. fortuitum,* (14) *M. smegmatis,* (15) *M. phlei,* (16) *M. africanum.*

### 5.2.6 Expression of the *M. bovis pel* gene

The PhoA+ phenotype of *M. smegmatis* (pM21), and Western blot analysis provided evidence that the *pel/phoA* gene fusion was expressed by recombinant *M. smegmatis* (Chapter 3). To demonstrate expression of the full-length (1404 bp) *pel* gene by recombinant *M. smegmatis*, Northern blot analysis was carried out using RNA samples prepared from the cosmid subclones. Total RNA from *M. smegmatis* (subclones 56/1 and 56/2) was transferred to a nylon membrane following electrophoresis and probed with a radiolabelled 234 bp restriction fragment of the *pel* gene. A broad band of approximately 1250 to 1700 nucleotides was observed in the lane containing an *M. smegmatis* (subclone 56/2) RNA sample (Figure 5.9B). The identified transcript is sufficiently large to encode the *pel* gene (1404 bp). The probe did not hybridise to RNA from the control sample. In other experiments, contaminating DNA in RNA samples was shown not to interfere with Northern blot results (data not shown), indicating that the probe hybridised to an RNA transcript of the *pel* gene.

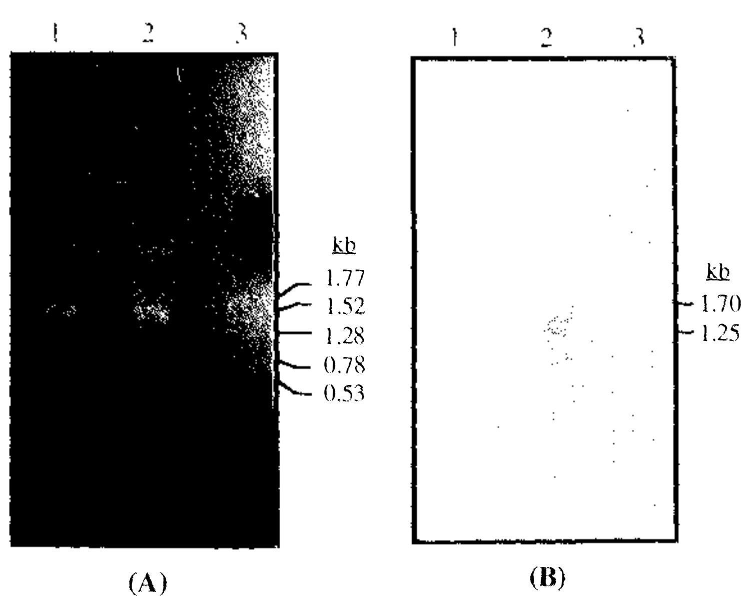



**Figure 5.9**

Expression of the *M. bovis pel* gene. (A) Ethidium bromide-stained agarose gel of total RNA from recombinant *M. smegmatis*. (B) Northern blot of total RNA from recombinant *M. smegmatis* probed with a 234 bp fragment from the *M. bovis* BCG insert of pM21 corresponding to part of the *pel* gene. Lanes 1, *M. smegmatis* (subclone 56/1); lanes 2, *M. smegmatis* (subclone 56/2); and lanes 3, RNA size standards.

### 5.2.7 Immunoscreening of CFs from recombinant *M. smegmatis* containing the cosmid subclones

Culture filtrates from the recombinant *M. smegmatis* cosmid subclones were tested *in vitro* for their ability to stimulate proliferation and IFN-γ-production by PBMCs taken from two *M. bovis* BCG-immunised cattle (animals 12 and 15). The immunised animals used in these experiments were selected on the basis of their high T cell reactivity against *M. smegmatis* (cosmid 56) CF (Chapter 4). Peripheral blood mononuclear cells from a non-immunised control animal (30) were also incubated with the CFs to monitor cross reactive T lymphocyte responses.

The lymphocyte proliferation and IFN-γ assay experiments were internally controlled by comparing responses between CF samples from the subclone 56/2 (containing the *pel* gene) and subclone 56/1 (containing a randomly selected similar sized DNA insert). Recombinant *M. smegmatis* containing subclones 56/1 and 56/2 were grown in minimal media and CFs prepared. Culture filtrates from the recombinant *M. smegmatis* and *M. bovis* BCG were assayed at a concentration of 2.0 µg per well (10 µg/ml). The proliferative responses to *M. bovis* BCG CF varied greatly between the two immunised animals (Table 5.1). The PBMCs from immunised animal 12 proliferated strongly in response to *M. bovis* BCG CF (SI = 275) compared to PBMCs from immunised animal 15 (SI of 5.8). No proliferation above background levels (SI = 0.5) was observed by PBMCs from the control in response to CF from *M. bovis* BCG. The proliferation SI's for PBMCs incubated with CF from *M. smegmatis* (subclone 56/2) were low (SI < 2) for all three animals and were not significantly different from those induced by CF from the negative control, recombinant *M. smegmatis* containing subclone 56/1 (Table 5.1).

| CF Added | Animal 12 (i) | | Animal 15 (i) | | Animal 30 (c) | |
|---|---|---|---|---|---|---|
| To PBMCs | mean cpm +/- stdev | SI | mean cpm +/- stdev | SI | mean cpm +/- stdev | SI |
| None | 663 +/- 180 | 1.0 | 2733 +/- 1007 | 1.0 | 1041 +/- 175 | 1.0 |
| Subclone 56/1 CF | 891 +/- 135 | 1.3 | 854 +/- 35 | 0.3 | 1264 +/- 426 | 1.2 |
| Subclone 56/2 CF | 1180 +/- 472 | 1.8 | 1189 +/- 406 | 0.4 | 1425 +/- 106 | 1.4 |
| *M. bovis* BCG CF | 182296 +/- 29370 | 275.0 | 15748 +/- 10077 | 5.8 | 572 +/- 85 | 0.5 |

**Table 5.1**

Proliferative responses of PBMC taken from *M. bovis* BCG-immunised (i) and non-immunised, control (c) cattle stimulated with 2.0 μg CF per well. Results are expressed as the mean counts per minute (cpm) of quadruplicate wells +/- 1 standard deviation (stdev), and as stimulation indices (SI's). Values outside the median cpm +/- 50% were disregarded.

SI = (mean cpm of stimulated wells) / (mean cpm of non-stimulated wells).

Culture filtrates from the recombinant *M. smegmatis* (cosmid subclones) were also tested for their ability to induce IFN-γ-production by PBMCs taken from the *M. bovis* BCG-immunised and control animals. Proliferation assay supernatants were harvested from five day cultures and assayed for the presence of IFN-γ using a sandwich ELISA. Optical density measurements were converted to units IFN-γ per ml using a recombinant IFN-γ standard curve.

Figure 5.9 shows the levels of IFN-γ produced by PBMCs from *M. bovis* BCG-immunised and non-immunised cattle in response to CF from *M. smegmatis* recombinants containing the two cosmid subclones. In contrast to the proliferative responses, IFN-γ-production by PBMCs from immunised animal 12 was higher in response to CF from recombinant *M. smegmatis* containing subclone 56/2 (10.9 units/ml) compared to CF from recombinant *M. smegmatis* containing subclone 56/1 (5.5 units/ml). Similar levels of IFN-γ were produced in response to CF from *M. smegmatis* (subclone 56/1) and *M. smegmatis* (subclone 56/2) by PBMCs from animals 15 (immunised) and 30 (control).

The responses to *M. bovis* BCG CF antigens correlated with the responses observed in the proliferation assay. Animal 12 responded very strongly to the *M. bovis* BCG CF antigens, producing 111.8 units/ml of IFN-γ, whereas animal 15 produced 27.3 units/ml. Peripheral blood mononuclear cells from the control animal (30) produced only 2.7 units/ml of IFN-γ in response to *M. bovis* BCG CF.

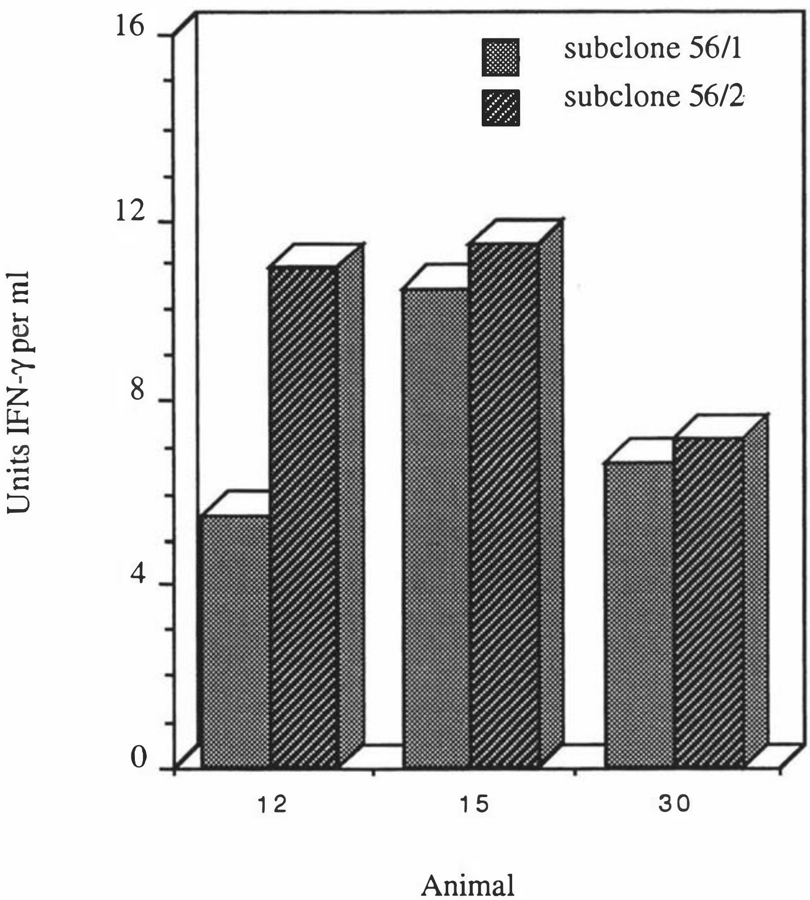



**Figure 5.10**

IFN-γ responses of PBMCs from *M. bovis* BCG-immunised animals (12 and 15) and a non-immunised, control animal (30) to CF from *M. smegmatis* recombinants containing the cosmid subclones. Results are expressed as the mean IFN-γ units/ml. The PBMCs from immunised animals 12, and 15 produced 111.8 and 27.3 IFN-γ units/ml, respectively, in response to *M. bovis* BCG CF (data not shown). The PBMCs from the control animal produced 2.7 IFN-γ units/ml in response to *M. bovis* BCG CF (data not shown).

## 5.3 Discussion

The immunoassays carried out on CF from selected recombinant *M. smegmatis* from the *M. bovis* cosmid library indicated that CF from *M. smegmatis* (cosmid 56) contained one or more potential T cell antigens (Chapter 4). DNA sequence and Southern blot analyses demonstrated that cosmid 56 contained a gene (*pel*) coding for an *M. bovis* putative exported lipoprotein with similarity to the product of an uncharacterised ORF in *M. leprae*.

The *M. bovis pel* gene was cloned, and its sequence determined and analysed. The *pel* gene was predicted to encode a 468 amino acid polypeptide. Cleavage of the putative 25 amino acid putative lipoprotein signal peptide would yield a mature Pel protein with a calculated molecular mass of 47.3 kDa. Database searches using the *M. bovis* Pel protein sequence revealed similarity to an *M. leprae* Pel homologue and MalE maltose-binding proteins of Gram negative and Gram positive bacteria. These sequence similarities raised the question as to whether Pel was a maltose-binding protein. In *E. coli,* the *malE* gene is part of the *malEFG* operon, one of two operons of the *malB* locus regulating maltose transport (Hofnung, 1974). The *malE* gene encodes the periplasmic maltose-binding protein, MalE. The *malF* and *malG* genes encode the cytoplasmic membrane components of the transport system, MalF and MalG, respectively. Other evidence for the potential involvement of Pel in maltose transport came from the observation that the *M. leprae pel* gene homologue mapped upstream of putative *malF* and *malG* genes. Hydropathy analysis of the *M. leprae* putative MalF and MalG proteins revealed several hydrophobic domains consistent with a cytoplasmic transmembrane location for these proteins. DNA sequence analysis revealed the start of a second ORF 53 bp downstream of the *M. bovis pel* gene. The deduced amino acid sequence of this ORF was 85% similar to the *M. leprae* putative MalF protein. The high level of conservation between the *M. leprae* and *M. bovis pel* and putative *malF* genes, together with the similar organisation of these

genes in *M. leprae* and *E. coli*, suggests that the *M. bovis pel* gene may be part of a mycobacterial *malEFG* operon.

The second operon in the *E. coli malB* locus regulating maltose and maltodextrin transport is divergently transcribed and comprises the *malK*, *lamB* and *malM* genes (Gilson *et al.*, 1986). The *malK* gene product, MalK, is located on the inner surface of the cytoplasmic membrane in association with MalF and MalG (Shuman and Silhavy, 1981). The *E. coli lamB* gene codes for the outer membrane protein LamB. MalM, the product of the *E. coli malM* gene, is a periplasmic protein of unknown function. Database searches with the *E. coli* LamB and MalM protein sequences revealed no similarity to mycobacterial proteins. However, database searches with the *E. coli* MalK sequence revealed strong similarity to a family of ATP-binding transport proteins, including the *M. leprae* putative glycerol-3-phosphate transport ATP-binding protein, UgpC. Complementation studies in *E. coli* indicate that UgpC and MalK are functionally exchangeable (Hekstra and Tommassen, 1993). Therefore, it is possible that the putative *ugpC* gene of *M. leprae* is in fact a cytoplasmic membrane-associated component of an *M. leprae* maltose and maltodextrin transport system. Interestingly, the *M. leprae ugpC* gene maps immediately downstream from *malG* in the same orientation. If *M. leprae ugpC* is involved in maltose transport, the *malB* locus in mycobacteria may contain a single operon with four genes; *malE (pel)*, *malF*, *malG*, and *malK (ugpC)*. This is highly speculative, but plausible in view of the genomic organisation of *M. leprae.* Although *malEFG* operons have been described for Gram positive organisms, a homologue of the MalK protein has not previously been described downstream of the *malEFG* operon.

Periplasmic binding-protein dependent transport systems, including those for maltose and maltodextrins, have been well characterised in Gram negative bacteria (Shuman, 1987). The substrate-binding components of these systems are exported through the cytoplasmic membrane into the periplasmic space. In Gram positive organisms the exported

substrate-binding components of analogous transport systems appear to be attached to the cytoplasmic membrane via an N-terminal lipo-amino acid anchor (Gilson *et al.*, 1988). A large number of lipoproteins involved in solute transport have been identified in Gram positive bacteria (reviewed in Sutcliffe and Russell, 1995). Five exported lipoproteins found in member species of the *M. tuberculosis* complex, MPT83, the 19 kDa antigen, and PstS-1, PstS-2, and PstS-3 have been characterised. While the function of MPT83 and the 19 kDa antigen is not known, the PstS antigens are a family of structurally homologous phosphate-binding proteins (Lefèvre *et al.*, 1997). Evidence for the role of PstS-1 in phosphate transport came from a recent study which demonstrated inhibition of phosphate uptake by *M. bovis* BCG using a monoclonal antibody directed against PstS-1 (Braibant *et al.*, 1996). The finding that the exported *M. bovis* Pel protein has a lipoprotein signal peptidase consensus sequence is consistent with Pel being the binding-protein component of a maltose and maltodextrin transport system. Although Pel is an exported protein, it is not clear if it would be released by the cell from its lipid anchor. The MPT83 putative lipoprotein is predominantly cell-associated as shown by immunoblotting cell lysates and CFs prepared from recombinant *M. smegmatis* containing the *mpt83* gene (Hewinson *et al.*, 1996). The three PstS proteins have also been located on the surface of *M. bovis* BCG by using flow cytometry (Lefèvre *et al.*, 1997). However, the 19 kDa and PstS exported lipoproteins of *M. tuberculosis* are found in the culture medium during growth, and have been shown to stimulate cell mediated immune responses in animal models (Young and Garbe, 1991). It remains to be determined if these lipoproteins are released from the mycobacterial cell *in vivo.*

Southern blot analysis of the distribution of *pel* among slow and fast growing mycobacterial species indicated that this gene may be present and moderately conserved in all mycobacteria. Under the hybridisation conditions used, the *pel* gene probe hybridised strongly to DNA from members of the *M. tuberculosis* complex, and weakly to DNA fragments from all other mycobacteria tested. Maltose transport systems are

ubiquitous among bacteria and the data from this experiment suggest that the regulatory genes are also conserved among mycobacteria.

Northern blot analysis provided evidence that the *pel* gene was expressed by recombinant *M. smegmatis* (subclone 56/2). The size of the RNA transcript detected by the *pel* gene probe (between 1250 and 1700 nucleotides) is consistent with the predicted length of the gene (1404 bp). Although no larger transcripts were identified by Northern blotting, this does not exclude the possibility that the *pel* gene is expressed as part of an operon. Processing of the RNA transcript of the maltose regulon has been reported in the Gram-positive bacterium *Streptococcus pneumoniae*, yielding an RNA molecule which includes the first gene in the operon and a larger fragment spanning the two downstream genes (Puyet and Espinosa, 1993).

The presence of the *pel* and putative *malF* genes, and the probable presence of *malG* and *malK* genes on the *M. bovis* cosmid subclone 56/2 make it unlikely that genes coding for other exported proteins would be present in the 8.3 kb DNA insert of subclone 56/2. A search of the available insert DNA sequence for ORFs did not reveal any with recognisable signal peptides. Therefore, it is probable that Pel would be the only *M. bovis*-encoded protein in CF from recombinant *M. smegmatis* (subclone 56/2).

Culture filtrate from recombinant *M. smegmatis* containing the *M. bovis pel* gene was assayed for its ability to induce proliferation and IFN-γ-production by PBMCs from *M. bovis* BCG-immunised and control cattle. Minimal proliferative responses were observed with CF from *M. smegmatis* containing the *M. bovis pel* gene with PBMCs from all animals tested. However, PBMCs from one of the *M. bovis* BCG-immunised cattle (animal 12) produced two-fold greater IFN-γ in response to CF from *M. smegmatis* (subclone 56/2), containing the *pel* gene, compared to CF from the *M. smegmatis* (subclone 56/1) negative control. The PBMCs from the second immunised animal (15) and the non-immunised control animal produced comparable levels of IFN-γ in response

to CF samples from the two *M. smegmatis* recombinants (cosmid subclones). The IFN-γ produced by PBMCs from the control animal in response to the recombinant *M. smegmatis* CFs may represent a cross-reactive response to proteins from environmental mycobacteria (e.g. *M. avium*). Alternatively, the CF proteins may be stimulating a subset of non-antigen specific lymphocytes. One possibility is that the CF antigens are stimulating innate T cells present in the PBMCs. These may include populations of γδ T cells and natural killer cells which are known to be involved in the innate immune responses (Trinchieri, 1990; Barnes *et al.*, 1994). In humans, γδ cells isolated from healthy tuberculin-negative individuals respond to *M. tuberculosis* demonstrating their innate capacity to recognise mycobacterial antigens without prior exposure (Pfeffer *et al.*, 1990). *M. tuberculosis*-reactive γδ T cell clones have been found to produce IFN-γ (Barnes *et al.*, 1994), and in young calves, γδ T cells make up 20-30% of all peripheral blood T cells (Wyatt *et al.*, 1994).

While the biological function of the *M. bovis* Pel protein remains to be proven, the proposed maltose transport function seems likely based on its sequence similarity with bacterial MalE maltose-binding proteins. Synthesis of these proteins is normally induced either in the presence of the substrate or derepressed under conditions of nutrient starvation (Chapon, 1982). Following phagocytosis, the ingested mycobacterium would require nutrients in order to survive. The ability to rapidly synthesise and secrete substrate-binding transport proteins would be essential for uptake of available nutrients. This would render these proteins as available targets for a host cell antigen specific response. Although the role of Pel in the immune response to *M. bovis* infection requires elucidation, it is possible that it could be a dominant antigen early after phagocytosis.

## 5.4 *Addendum*

The nucleotide sequence of the *M. bovis pel* gene and flanking sequences (shown in Figure 5.2) was used to search the Sanger Centre *M. tuberculosis* DNA database for similarities. The *M. bovis pel* gene and flanking sequences were 99.9% similar to an incomplete *M. tuberculosis* sequence deposited in the database in mid-September, 1997. To determine if the *M. tuberculosis* genome encoded homologues of *M. leprae* MalF, MalG and UgpC, the amino acid sequences of these proteins were used to search the translated *M. tuberculosis* DNA database. The translated *M. tuberculosis* DNA sequence showed greater than 81% identity and 88% similarity to the amino acid sequences of the three *M. leprae* proteins. The three *M. tuberculosis* ORFs, encoding the MalF, MalG and UgpC proteins, mapped immediately downstream of the *M. tuberculosis pel* gene homologue. The order of these ORFs was the same as the corresponding ORFs in *M. leprae* (see Figure 5.7). The finding that the genomic arrangement of *pel*, *malF*, *malG* and *ugpC* is highly conserved between *M. leprae* and *M. tuberculosis* provides supporting evidence for a proposed maltose transport locus.

# Chapter 6:

# Immune Responses Of *M. bovis* Challenged Cattle To Culture Filtrate From Recombinant *M. smegmatis* Expressing The *pel* Gene

## 6.1 Abstract

Culture filtrate from recombinant *M. smegmatis* containing the *pel* gene (cosmid 56 and subclone 56/2) contains antigens that are recognised by *M. bovis* BCG-immunised cattle. To extend this observation, immune responses of PBMCs from four *M. bovis* challenged and four *M. bovis* BCG vaccinated / *M. bovis* challenged cattle to CF from *M. smegmatis* (subclone 56/2) were assessed. When compared to the subclone 56/1 CF control sample, CF from *M. smegmatis* (subclone 56/2) induced greater immune responses by PBMCs from three challenged cattle (animals 25, 37 and 64) and two vaccinated / challenged cattle (animals 72 and 75). This provides further evidence that CF from recombinant *M. smegmatis* expressing the *pel* gene contains a T cell antigen. The immune responses did not appear to correlate with lesion formation or protection among the animals tested.

## 6.2 Introduction

Studies assessing the ability of *M. bovis* BCG vaccination to induce protective immunity in cattle have shown that, under carefully controlled conditions, vaccination with a low dose can afford a significant level of protection against virulent *M. bovis* challenge (Buddle *et al.*, 1995a). It has also been shown that T cells from calves vaccinated with viable *M. bovis* BCG are able to recognise a wide spectrum of *M. bovis* secreted proteins (Gulle *et al.*, 1995). Natural hosts for tuberculosis such as cattle therefore offer a good model system to identify antigens which might serve as diagnostic agents by examining the antigen-specific lymphocyte responses of vaccinated and *M. bovis*-infected animals.

Vaccination of cattle with *M. bovis* BCG potentially complicates the use of skin testing as a diagnostic tool, and as yet there is no single, rapid and cost-effective diagnostic test that can distinguish vaccinated and vaccinated / infected animals. This has lead to studies to evaluate the use of single antigens as potential diagnostic agents or as components of a novel vaccine. In chapter 4 of this work, CFs from selected *M. smegmatis* recombinants (identified by hybridisation to insert DNA from PhoA+ clones) were screened for their potential as T cell antigens in *M. bovis* BCG-immunised cattle. The screening system detected one or more bovine T cell antigens in CF from *M. smegmatis* (cosmid 56), which contained the *M. bovis* BCG *pel* gene. The *pel* gene was subcloned into pSUM40 and CF from recombinant *M. smegmatis* expressing the subcloned *pel* gene tested in immunoassays using PBMCs from *M. bovis* BCG-immunised cattle. The level of IFN-γ produced by PBMCs from one immunised animal in response to CF from recombinant *M. smegmatis* expressing the *pel* gene was two-fold greater than the level induced by CF from *M. smegmatis* containing a plasmid with randomly selected DNA insert of similar size. This chapter extends these studies to examine responses to CF from recombinant *M. smegmatis* expressing the *pel* gene by PBMCs from *M. bovis* challenged and *M. bovis* BCG vaccinated / *M. bovis* challenged cattle.

A rigorously controlled *M. bovis* BCG cattle vaccination trial has recently been carried out at AgResearch Wallaceville by Dr. Bryce Buddle. Groups of animals were vaccinated with low dose (approximately $10^5$ c.f.u.) *M. bovis* BCG or experimentally infected with *M. bovis*. At four to eight weeks post-vaccination with *M. bovis* BCG some of these animals were challenged intratracheally with *M. bovis*. There were four different groups of animals in the trial: (1) control-unvaccinated, (2) *M. bovis* BCG vaccinated, (3) *M. bovis* challenged, and (4) *M. bovis* BCG vaccinated / *M. bovis* challenged. Blood samples were made available to our laboratory to test the responses (T lymphocyte proliferation and IFN-γ-production) against CFs prepared from recombinant *M. smegmatis* containing *M. bovis* genes, including *pel*. The aim of the experiments described in this chapter was to determine if CF from recombinant *M. smegmatis* expressing the *pel* gene could be recognised by PBMCs from *M. bovis* challenged animals. It was also of interest to determine if the CF could distinguish between the *M. bovis* challenged and *M. bovis* BCG vaccinated / *M. bovis* challenged groups of animals on the basis of differing PBMC proliferation and IFN-γ responses.

## 6.3 Results

**Immune recognition of CF from recombinant *M. smegmatis* expressing the *pel* gene by PBMCs from *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged cattle.**

Blood samples from four *M. bovis* challenged, and four *M. bovis* BCG vaccinated / challenged cattle, collected 15 weeks post challenge, were used in experiments to test lymphocyte responses to CF from recombinant *M. smegmatis* expressing the *pel* gene. Blood samples from the other two groups of cattle (unvaccinated controls and *M. bovis* BCG vaccinated) were not available for comparison at this time.

Approximately 2 x $10^5$ PBMCs were stimulated with 5.0 μg/well CF samples derived from the recombinant *M. smegmatis* (cosmid subclones). Proliferative responses to CF from *M. smegmatis* harbouring subclone 56/2 (expressing the *pel* gene) were compared to responses induced by CF from *M. smegmatis* harbouring subclone 56/1 (containing a randomly selected 10 kb *M. bovis* DNA insert).

The proliferative responses of the unvaccinated, *M. bovis* challenged animals to CFs from the cosmid subclones are shown in Figure 6.1. The PBMCs from three of the four challenged cattle (animals 25, 37 and 64) were most responsive to the *M. smegmatis* (subclone 56/2) CF sample. The mean thymidine incorporation by PBMCs from animal 25 stimulated with CF from subclone 56/2 was 5.9-fold higher than with CF from subclone 56/1, and 2.4-fold higher than incorporation by unstimulated cells. With animal 37, the proliferation induced by CF from *M. smegmatis* (subclone 56/2) was nearly double (1.8-fold higher) that induced by the control CF sample. The proliferative response of PBMCs from animal 64 to CF from *M. smegmatis* (subclone 56/2) was 2.5-fold higher than with CF from *M. smegmatis* (subclone) 56/1. Animal 30 was the only unvaccinated, *M. bovis* challenged animal to respond similarly to the two CF samples.

The proliferative responses of PBMCs from the *M. bovis* BCG vaccinated / *M. bovis* challenged animals to CFs from the cosmid subclones are shown in Figure 6.2. One of the four vaccinated / challenged cattle (animal 75) was highly responsive to the *M. smegmatis* (subclone 56/2) CF sample. The mean thymidine incorporation was 6.7-fold higher in the presence of CF from subclone 56/2 than when stimulated with CF from subclone 56/1. The levels of proliferation by PBMCs from the other three cattle (animals 55, 59 and 72) were not significantly different between the two recombinant *M. smegmatis* CF samples. However, the magnitude of these responses relative to the background response (proliferation in the absence of added antigen) showed considerable variation. Lymphocyte proliferation induced by the recombinant *M. smegmatis* CF samples was approximately 2- and 4-fold greater than the background proliferation for animals 59 and 72, respectively. In contrast, the proliferation of PBMCs from animal 55, when stimulated with recombinant *M. smegmatis* CFs, was approximately 165-fold greater than proliferation in the absence of added antigen.

The mean background levels of thymidine incorporation by PBMCs from the eight animals (Figures 6.1 and 6.2) varied considerably, ranging from 386 cpm (animal 30) to 9,632 cpm (animal 64). Similarly, the proliferative responses to the negative control CF sample (*M. smegmatis* harbouring subclone 56/1) showed large variation between animals, ranging from 744 cpm (animal 25) to 92,979 cpm (animal 55).

In addition to examining the proliferative responses to CF samples from the recombinant *M. smegmatis* (cosmid subclones), the CFs were tested for their ability to induce IFN-$\gamma$-production by PBMCs from *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged cattle. Proliferation assay supernatants were harvested from five day cultures, pooled, and assayed in duplicate for the presence of IFN-$\gamma$ using a sandwich ELISA. The optical densities (OD) of the duplicate samples were measured at 450 nm and the averages calculated.

Figure 6.3 shows the levels of IFN-γ produced by PBMCs from three unvaccinated, *M. bovis* challenged cattle in response to CF from *M. smegmatis* recombinants containing subclones 56/1 and 56/2. Supernatants from the fourth challenged animal (animal 37) were unavailable for assay. The IFN-γ-production by PBMCs from challenged animal 25 was significantly higher in response to CF from recombinant *M. smegmatis* containing subclone 56/2 ($OD_{450}$ > 2.7) compared to CF from recombinant *M. smegmatis* containing subclone 56/1 ($OD_{450}$ = 0.56). As found in the proliferation assay, animal 25 did not respond above background to CF from *M. smegmatis* (subclone 56/1). Similar levels of IFN-γ were produced in response to CF from the two *M. smegmatis* recombinants by PBMCs from animals 30 and 64. However, animal 64, which responded more strongly to the subclone 56/2 CF sample in the proliferation assay, produced IFN-γ levels above the range measurable by ELISA in response to both CF samples, so possible differences in responses would not be not detected. There was insufficient culture supernatant to repeat the assay using diluted samples.

The IFN-γ produced by PBMCs from the four *M. bovis* BCG vaccinated / *M. bovis* challenged animals in response to CFs from the cosmid subclones are shown in Figure 6.4. The IFN-γ-production by PBMCs from animal 75 was higher in response to the *M. smegmatis* (subclone 56/2) CF sample ($OD_{450}$ = 1.76) compared to the *M. smegmatis* (subclone 56/1) CF sample ($OD_{450}$ = 0.82). The response induced by CF from recombinant *M. smegmatis* containing subclone 56/2 was also higher than the response to CF from subclone 56/1 for animal 72. However, unstimulated cells from this animal also produced very high levels of IFN-γ ($OD_{450}$ = 1.89). With animals 55 and 59, similar levels of IFN-γ were produced by PBMCs in response to CF from subclones 56/1 and 56/2. The levels of IFN-γ produced by PBMCs from animal 55 in response to the recombinant *M. smegmatis* CF samples were above the range measurable by ELISA.

These results demonstrate that, under the conditions used, CF from recombinant *M. smegmatis* expressing the *pel* gene could not clearly distinguish between PBMCs from the *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged groups of cattle.

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At the conclusion of the *M. bovis* BCG vaccination trial, Dr. Bryce Buddle provided information on the infection status of animals 25, 30, 37, 64, 55, 59, 72 and 75. The cattle were slaughtered approximately four months post-challenge and subjected to extensive post-mortem examination at AgResearch, Wallaceville. Animals were scored for visible lesions typical of tuberculosis infections, and culture positivity. The non-vaccinated / *M. bovis* challenged cattle (animals 25, 30, 37 and 64) all showed macroscopic lesions and were culture positive at post-mortem. All four *M. bovis* BCG vaccinated / *M. bovis* challenged cattle (animals 55, 59, 72 and 75) were culture positive, although only animals 59 and 75 showed visible lesions. Animals 55 and 72 showed no evidence of lesions typical of tuberculosis infections.

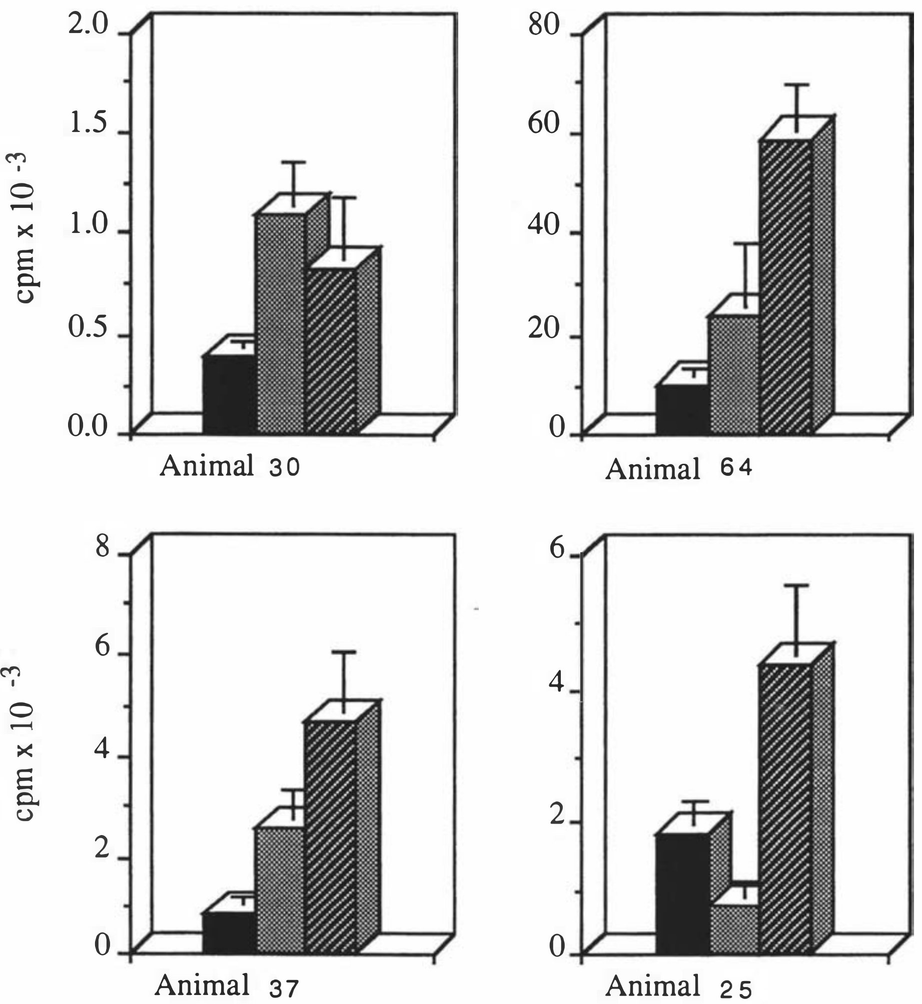


**Figure 6.1**

Proliferation of PBMCs from four *M. bovis* challenged cattle in response to CF from recombinant *M. smegmatis* containing subcones 56/1 ▩ and 56/2 ▨. As a control, proliferation in the absence of added antigen is also shown ■. The CFs were assayed at a concentration of 5.0 µg/well.

Proliferation of cells was measured by incorporation of [$^3$ H] thymidine. Results are expressed as the mean counts per minute (cpm) x$10^{-3}$ of quadruplicate sample wells. Error bars represent one standard deviation.

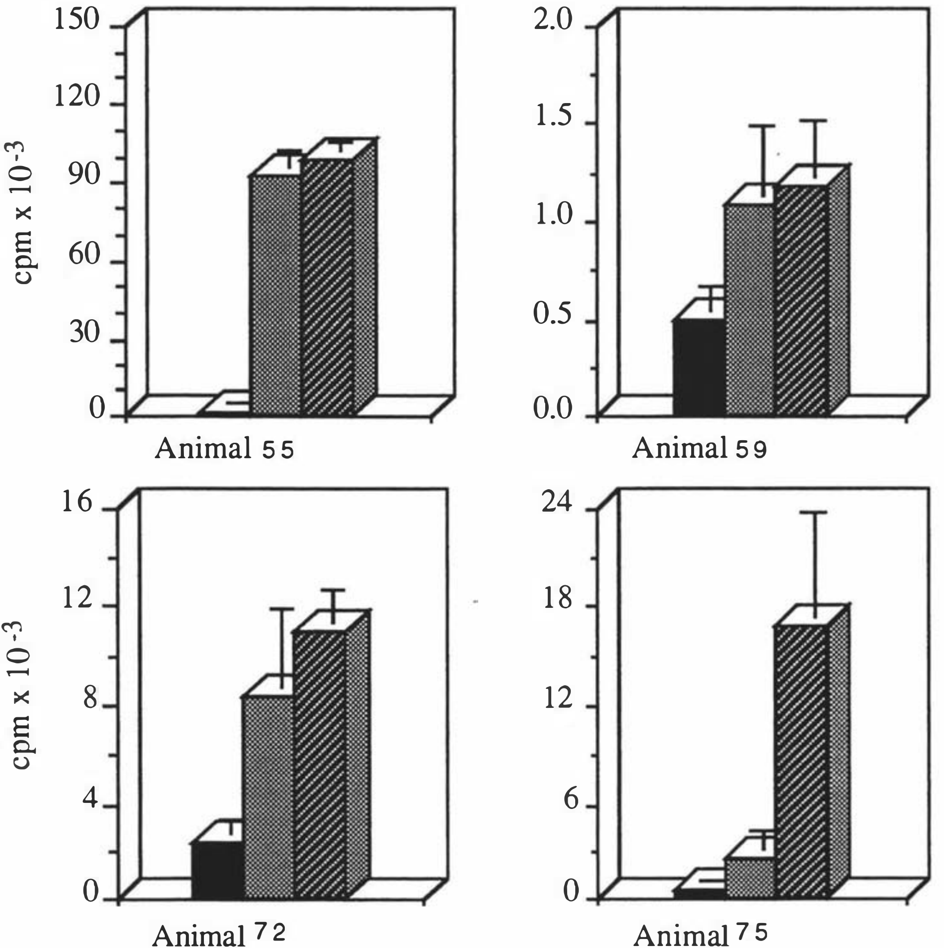


**Figure 6.2**

Proliferation of PBMCs from four *M. bovis* BCG vaccinated / *M. bovis* challenged cattle in response to CF from recombinant *M. smegmatis* containing subcones 56/1 ▩ and 56/2 ▨. As a control, proliferation in the absence of added antigen is also shown ■. The CFs were assayed at a concentration of 5.0 μg/well. Proliferation of cells was measured by incorporation of [$^3$H] thymidine. Results are expressed as the mean counts per minute (cpm) x10$^{-3}$ of quadruplicate sample wells. Error bars represent one standard deviation.

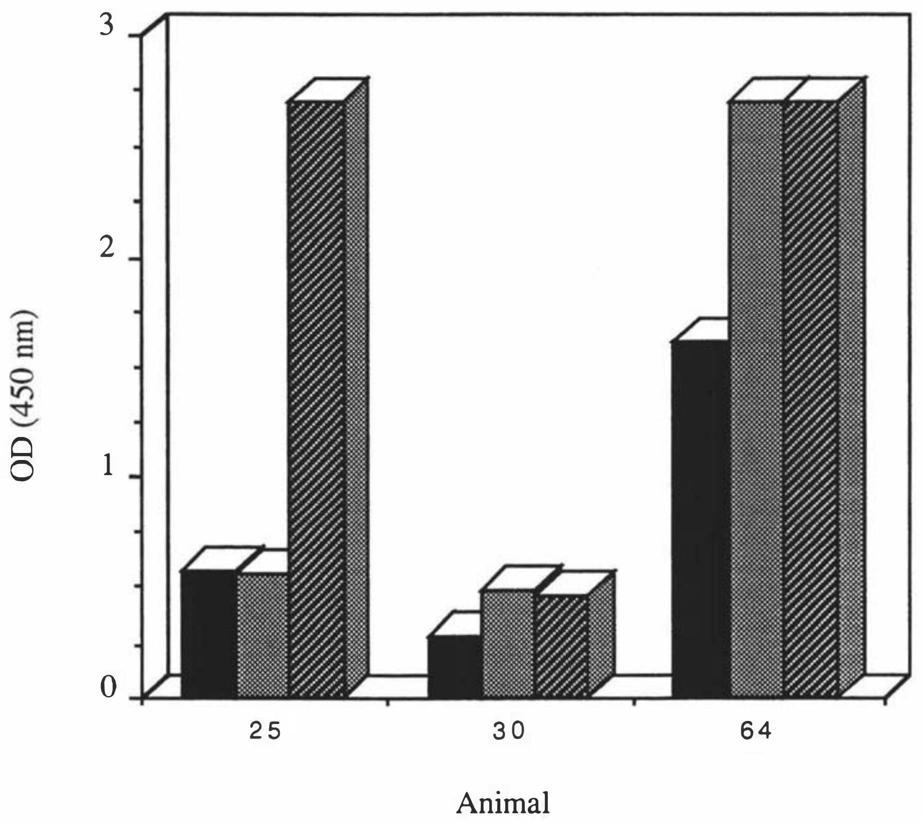


**Figure 6.3**

IFN-γ responses of PBMCs ($2x10^5$) from three *M. bovis* challenged cattle to CF prepared from recombinant *M. smegmatis* containing subclones 56/1 and 56/2. IFN-γ-production in the absence of added is antigen is also shown. The CFs were assayed at a concentration of 5.0 μg/well. IFN-γ was detected in culture supernatants using a sandwich ELISA. The optical densities (OD's) of samples were measured at 450 nm (maximum OD=2.7).

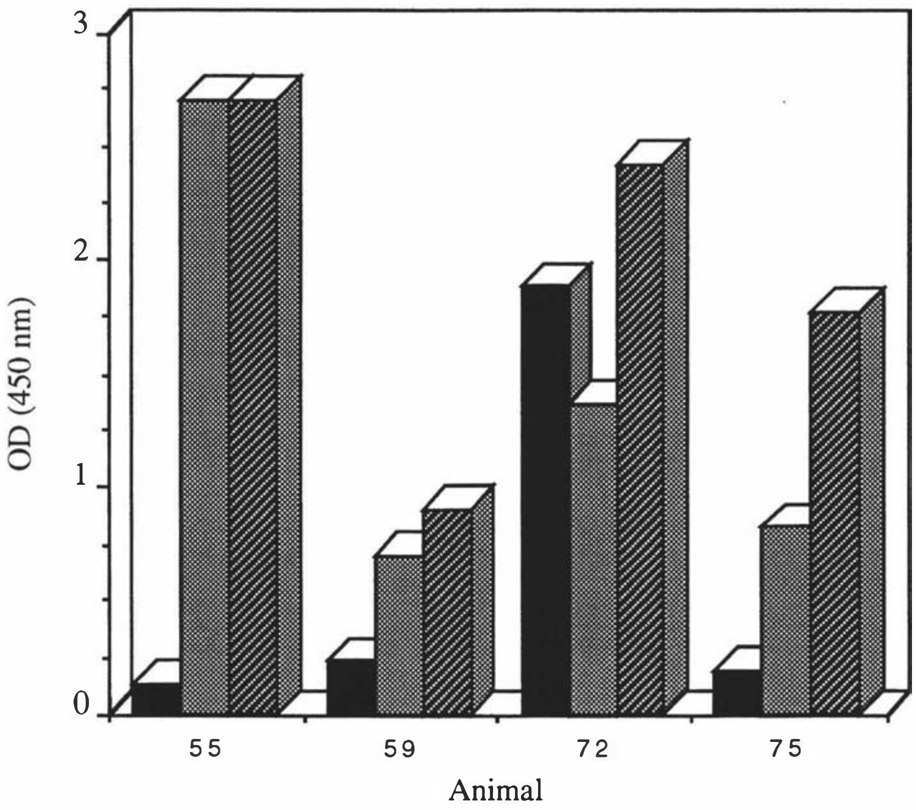


**Figure 6.4**

IFN-γ responses of PBMCs from four *M. bovis* BCG vaccinated / *M. bovis* challenged cattle to CFs from recombinant *M. smegmatis* containing subclones 56/1 ▩ and 56/2 ▨. The production of IFN-γ in the absence of added is antigen is also shown ■. The CFs were assayed at a concentration of 5.0 μg/well. IFN-γ was detected in culture supernatants using a sandwich ELISA. The optical densities (OD's) of samples were measured at 450 nm (maximum OD= 2.7).

## 6.4 Discussion

Current diagnosis for bovine tuberculosis is carried out by intradermal testing with purified protein derivative (PPD). The PPD preparation is a poorly defined mycobacterial extract containing numerous antigens which can cause a lack of specificity. Comparative intradermal testing has improved the specificity of diagnosis by taking into account cross-reactive responses to environmental mycobacteria such as *M. avium* (Monaghan *et al.*, 1994). An alternative diagnostic test, based on *in vitro* measurement of IFN-γ in response to PPD has been developed that is both rapid and highly sensitive (Wood *et al.*, 1991). Results demonstrating the protective capacity of *M. bovis* BCG in bovines (Buddle *et al.*, 1995a) have opened the possibility that *M. bovis* BCG could be used as a vaccine to control bovine tuberculosis. However, if the *M. bovis* BCG vaccine is to be used in cattle, it will be necessary to distinguish between *M. bovis* infected and *M. bovis* BCG vaccinated cattle. In recent years, progress has been made in evaluating the diagnostic potential of single antigens, including those specific to member species of the *M. tuberculosis* complex (Wood *et al.*, 1992; Pollock and Andersen, 1997a)

The results from the immunoassays using CF preparations from recombinant *M. smegmatis* containing the *pel* gene (cosmid 56 and subclone 56/2) indicate that the CF was recognised by *M. bovis* BCG-immunised animals. The CF from *M. smegmatis* (subclone 56/2) were then used to compare the responses of *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged animals. At 15 weeks post-challenge, under the conditions used in these experiments, CF from recombinant *M. smegmatis* expressing the *pel* gene could not clearly distinguish between PBMCs from the *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged groups of cattle. When compared to the subclone 56/1 CF sample, CF from *M. smegmatis* (subclone 56/2) induced greater immune responses by PBMCs from three challenged animals and two vaccinated / challenged animals. The immune responses did not appear to correlate with lesion formation or protection among the animals tested. It is possible that the concentrations of antigens in

the recombinant *M. smegmatis* CF were outside the optimal range required for detecting discriminatory immune responses. In an independent study carried out in this laboratory, dose-response experiments demonstrated that the immune responses from PBMCs are optimal within a narrow range of concentrations of the recombinant *M. smegmatis* CF (Carpenter *et al.*, 1995). A study measuring proliferative responses to purified *M. bovis* antigens by PBMCs from experimentally infected cattle reported the optimal concentration range to be 2-10 μg/ml (Fifis *et al.*, 1994). The concentration of the Pel protein would be considerably less than this in the 5.0 μg/ml *M. smegmatis* (subclone 56/2) CF sample used in the experiments described in this chapter.

The *M. tuberculosis* complex-specific ESAT-6 protein has also been used as a single antigen to assess the immune responses in cattle experimentally infected with *M. bovis* (Pollock and Andersen, 1997a). The magnitude of the immune responses to a low molecular mass (<10 kDa) fraction of *M. tuberculosis* CF (containing ESAT-6) from *M. bovis* infected *M. bovis* animals varied over time, post-infection (Pollock and Andersen, 1997b). This variation in the magnitude of responses over time has also been reported in a study measuring immune responses to purified *M. bovis* antigens by experimentally infected cattle over a 3 year time course (Fifis *et al.*, 1994). One of the purified antigens, MPB70, induced at least a 10-fold difference in IFN-γ-production by PBMCs at different stages of infection. The CF from recombinant *M. smegmatis* expressing the *pel* gene was tested at a single time point, 15 weeks post infection, which may not be the optimal time for distinguishing between the immune responses of *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged cattle. The Fifis study demonstrated that different antigens were immunodominant at different stages of infection, suggesting that a combination of antigens may be required for use in a cellular diagnostic test. The results of this study indicate that CF from recombinant *M. smegmatis* expressing the *pel* gene is recognised by PBMCs from both *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged animals. Given the changing profile of immunodominance during *M. bovis* infection, it

would be necessary to test different concentrations of the Pel protein over a period of many months to evaluate its potential as an immunodiagnostic reagent.

# Chapter 7:

## Discussion Overview

The aim of the work presented in this thesis was to apply a novel two-step approach to the identification of *M. bovis* exported proteins with potential T lymphocyte reactivity. In combining the *phoA*-fusion methodology with cosmid library T cell screening, the impact of the limitations imposed by each of these systems was minimised, and their individual benefits combined to form a powerful strategy for identifying novel exported *M. bovis* T cell antigens. The identification of the *M. bovis* Pel protein demonstrates the power of combining these strategies to identify potential novel T cell antigens.

The development, in recent years, of heterologous expression systems has greatly enhanced the ability to carry out genetic manipulation and analysis in mycobacteria. Expression of genes from slow-growing, pathogenic mycobacteria, such as *M. bovis*, in the fast-growing *M. smegmatis* allows for gene expression signals to be recognised in a related host. In addition, processing and post-translational modification pathways are likely to be conserved and the structural integrity of the recombinant protein may be similar, if not identical, to the native form. Several *M. tuberculosis* and *M. bovis* secreted proteins have been expressed in *M. smegmatis* and purified, including the 19 kDa antigen, MPB64 and MPT64, and antigens 85-A and 85-B (Garbe *et al.*, 1993; Tasaka *et al.*, 1995; Roche *et al.*, 1996; Harth *et al.*, 1997) These recombinant proteins displayed similar antigenicity to the native form of the proteins and appeared identical on SDS-polyacrylamide gels.

The development of *phoA*-fusion technology has provided a rapid technique to identify mycobacterial exported protein genes (Lim *et al.*, 1995). This represents a particularly useful advance in mycobacterial technology as extracellular proteins are the focus of much attention as targets of the cell-mediated immune response. In the present study, the *phoA*-fusion strategy was used to identify *M. bovis* BCG genes coding for exported proteins. Analysis of the deduced amino acid sequences of five of the PhoA fusion

proteins revealed that three fusions contained recognisable signal peptides. This suggested that the *phoA*-fusion technology was identifying proteins exported by the general secretory pathway (i.e. signal peptide-dependent export) as well as proteins exported by other mechanisms. Evidence to support the existence of a signal peptide-independent export pathway comes from another study using *phoA*-fusion technology, in which an enzymatically active PhoA fusion with the *M. tuberculosis* Des protein showed no structural similarity to known signal peptides at its N-terminal region (Jackson *et al.*, 1997). The SOD, L-alanine dehydrogenase, and ESAT-6 proteins also lack recognisable signal peptides, yet are present in short-term mycobacterial culture filtrates (Zhang *et al.*, 1991; Andersen *et al.*, 1992a; Sϕrensen *et al.*, 1995). The signals required for protein export by a signal peptide-independent export pathway are not known, although it is possible that these might be contained within the mature protein sequence.

The *M. bovis BCG* DNA inserts from ten recombinant plasmids expressing enzymatically active PhoA fusion proteins were partially sequenced and used to search databases for similarities to known genes. Database searches revealed similarities to a family of high temperature requirement (HtrA) serine proteases, a putative 1.4 kb lipoprotein gene (*pel*) from *M. leprae*, and uncharacterised *M. tuberculosis* ORFs. All ten sequences represent potentially novel exported protein genes.

Analyses of enzymatically active PhoA fusion proteins on Western blots suggested that the PhoA fusion proteins may have been post-translationally modified, possibly by proteolytic degradation. The *E. coli* periplasmic protease DegP has been shown to degrade PhoA fusion proteins to yield smaller products with PhoA activity (Strauch and Beckwith, 1988). Mycobacterial homologues of the DegP protein have been identified (Cameron *et al.*, 1994; Wu *et al.*, 1997), including one in this study; HtrA, partially encoded by the plasmid pM2.

Although the *phoA*-fusion technology has been successfully applied in this study to identify *M. bovis* BCG genes coding for exported proteins, there are limitations to its usefulness.

(1) The cloning of random fragments increases the possibility that non-contiguous DNA fragments will be cloned in the same DNA vector resulting in hybrid genes.

(2) The difficulty in obtaining full length, stable fusion products in *E. coli* and *M. smegmatis*.

(3) Characterisation of sequences identified using *phoA* fusions requires locating the full-length genes.

To overcome these limitations, this technology can be combined with other complementary systems for identifying exported protein antigens.

By using the insert DNA sequences from PhoA+ clones to screen an *M. bovis* cosmid library in *M. smegmatis* for full-length exported protein genes, the advantages of *phoA*-fusion and recombinant cosmid libraries are combined. The large insert sizes present in cosmid libraries increase the probability of locating full-length genes and their regulatory regions on a single cosmid. The large insert sizes also enable entire operons to be expressed, allowing identification of genes which might not be expressed in conventional expression libraries. By working with full-length recombinant proteins rather than protein fusions, protein instability is less of a problem. Recombinant *M. bovis* secreted antigens in CFs prepared from an *M. bovis* cosmid library in *M. smegmatis* are likely to be similar to the native proteins in terms of processing, post-translational modifications, and T cell recognition. A novel strategy for identifying secreted T cell antigens was described by Averill and colleagues, who screened culture filtrates from an *M. bovis* BCG cosmid library in *M. smegmatis* with PBMCs from human tuberculin-positive donors (Averill *et al.*, 1993). Three potentially novel antigens were identified following screening of CF prepared from 10% of the cosmid library. A similar approach has been successfully used in this laboratory to identify bovine T cell antigens (Carpenter *et al.*, 1995).

The screening of CFs from the recombinant *M. bovis* cosmid library provided a sensitive and rapid method for detecting a potential T cell antigen encoded by the *pel* gene. The *M. bovis pel* gene codes for a putative exported lipoprotein. The *pel* gene was subcloned from cosmid 56 of the *M. bovis* cosmid library and its sequence determined and analysed. Database searches using the deduced amino acid sequence of Pel revealed similarities to an *M. leprae* putative exported lipoprotein and a family of MalE maltose-binding proteins. In other bacteria, the *malE* gene is part of one of two operons regulating the transport of maltose and maltodextrins (Hofnung, 1974). Analysis of DNA sequences adjacent to the *M. bovis pel* gene and its *M. leprae* homologue, and their use in database searches, suggested that *pel* may be part of an operon involved in the transport of maltose and maltodextrins in mycobacteria. The synthesis of substrate-binding proteins, such as MalE, is normally induced in the presence of the substrate or derepressed under conditions of nutrient starvation (Chapon, 1982). However, the function of these mycobacterial transport proteins synthesised *de novo* is unclear. Lipoproteins make up only a subset of secreted proteins of mycobacteria and may be important in mediating interactions between the mycobacteria and the host cell. If essential nutrients are in limited supply within the host cell then competition with high-affinity binding transporter proteins exported by the mycobacterium might reduce the availability of the same nutrients for metabolism by the host. Conversely, a targeted immune response against such transporter proteins would reduce the fitness of the bacterium and restore the availability of nutrients to the host.

Preliminary evidence from this study indicates that the Pel protein is recognised by antigen-specific lymphocytes from *M. bovis* BCG-immunised animals. The PBMCs taken from *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged cattle also recognised CF potentially containing the Pel protein in *in vitro* immunoassays. However, the magnitude of the responses were not sufficient to distinguish between *M. bovis* challenged and *M. bovis* BCG vaccinated / challenged animals. Whether these responses

affect the viability of *M. bovis* / *M. bovis* BCG inside macrophages remains to be determined. The development of improved targeted gene disruption protocols in mycobacteria should greatly increase the understanding of the role of such exported proteins in pathogen / host interactions. In addition, a deeper understanding of the nature of the immune responses directed towards exported proteins will further the knowledge of how host cells successfully control intracellular parasites. It should also lead to the development of novel vaccines and therapeutic agents designed to eliminate important diseases, such as tuberculosis, in humans and cattle.

# Appendices:

## Appendix 1: Reagents

TE buffer (Sambrook *et al*. 1989)

10 mM Tris

1 mM EDTA

Adjust to pH 8.0 with concentrated HCl. Sterilise by autoclaving.

5 x SDS gel loading buffer (Sambrook *et al*. 1989)

250 mM Tris-HCl (pH 6.8)

500 mM dithiothreitol

10% SDS (electrophoresis grade)

0.5% bromophenol blue

50% glycerol

Store in aliquots at -20°C.

Agarose gel loading buffer (type III) (Sambrook *et al*. 1989)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in water

Store at -20°C.

20 x SSC (Sambrook *et al*. 1989)

3 M NaCl

300 mM sodium citrate

Adjust pH to 7.0 with 10 M NaOH. Sterilise by autoclaving.

10 x MOPS running buffer (Rueger *et al*, 1996)

0.2 M MOPS (pH 7.0)

50 mM sodium acetate

10 mM EDTA

Make up in sterile DEPC-treated water and autoclave.

RNA loading buffer (Rueger *et al*, 1996)

250 μl formamide, deionized

83 μl formaldehyde, 37% (w/v)

50 μl 10 x MOPS buffer

0.01% (w/v) bromophenol blue

Prepare fresh solution for each use.

1% agarose-MOPS formaldehyde gel composition (40 ml gel)

0.4 g agarose

28.8 ml DEPC-treated water

Melt agarose and cool to 60°C. Add the following:

5 μl 10 mg/ml ethidium bromide (in DEPC-treated water)

4 ml 10 x MOPS running buffer

7.2 ml 12.3 M formaldehyde

Leave to set for at least 30 mins in fume hood before use.

## Appendix 2: Oligonucleotide Primers

### (A) PCR primers used to amplify *M. bovis* secreted protein genes

| Target Gene | PCR Primer Sequences | Gene Reference |
|---|---|---|
| *85-A* | (1) TGG ATG CGT TGA GAT GAG GA | De Wit *et al.* (1990) |
| | (2) TAG AAG CTT GAC TGG CCA C | |
| *85-B* | (1) GCT GCC AGA CTT ACA AGT GG | Matsuo *et al.* (1988) |
| | (2) TGG AAC TTC AGG TTG CTG C | |
| *85-C* | (1) ACG ACA TTC GAA CTG AGC G | Content *et al.* (1991) |
| | (2) AGC CGT TGT AGT CAT CCT GG | |
| *mpb70* | (1) GAA CAA TCC GGA GTT GAC AA | Terasaka *et al.* (1989) |
| | (2) AGC ACG CTG TCA ATC ATG TA | |
| *mpb64* | (1) CAG GCA TCG TCG TCA GCA GC | Yamaguchi *et al.* (1989a) |
| | (2) GTG ATT GGC TTG CGA TAG GC | |
| *esat-6* | (1) ACA TGA CAG AGC AGC AGT GG | Søfrensen *et al.* (1995) |
| | (2) TGA CAA CCT CTC AGA GTG CG | |
| *apa* | (1) TAA GCC TCG TCA GCT ATC CG | Laqueyrerie *et al.* (1995) |
| | (2) CCA TTG ACC AGG TTG ACT CC | |
| *pstS (pab)* | (1) AGA GGT ATC CGA ACG TCA CG | Andersen and Hansen (1989) |
| | (2) TGA ACA AGA AGG TGT CAC CG | |
| *sodA* | (1) ACG TCA ATC ACA CCA TCT GG | Zhang *et al.* (1991) |
| | (2) TGT ACT GCA GGT AGA AGG CG | |
| *19 kDa Ag* | (1) CAA TTC CGT GTA ACC ACG G | Ashbridge *et al.* (1989) |
| | (2) GAA TCG TCA CGG TGA TTG C | |

## Appendix 2: Oligonucleotide Primers

### (B) Oligonucleotide primers used to sequence the 1521 base pair *M. bovis* BCG DNA insert of pM21

T* = transcriptional terminator sequence

# = refer pM21 insert DNA sequence, Appendix 1.1.

NB: 'R' primer sequences hybridise to the complementary DNA strand.

| Sequencing Primer | Oligonucleotide Primer Sequence | Nucleotides To Which Primer Hybridises# |
|---|---|---|
| PJEM6 | GCA GTA ATA TCG CCC TGA GCA GC | T* sequence of pJEM11 |
| PJEM7 | TTA ARR GGG GAC CCT AGA GGT CC | *phoA* sequence of pJEM11 |
| MB21R | TTG GCC TTG CTC ATC GAG GC | 110-129 of insert |
| MB21R4 | GAG CAA CGG TTA CAG CTG GC | 1184-1203 of insert |
| MB21R5 | ATG TGG TCA TGA GTC GCG GG | 960-979 of insert |
| MB21R6 | CTG ATC ACC TCT GCA GTG CC | 792-811 of insert |
| MB21R8 | GTT CAT CGG GTT GGT GCT CG | 707-726 of insert |
| MB21R12 | GAG TAG CTG GAC CTG ACT GG | 345-364 of insert |
| MB21R13 | CAG CAG GTG ACC ATC GTT GG | 594-613 of insert |
| MB21F | GGT GCT GAT CAA CTC GTC GG | 1391-1372 of insert |
| MB21F2 | CAA GCT GAC CTG CGC AAT GG | 1168-1149 of insert |
| MB21F4 | TCG TCA ACG CCA CCA GTA CG | 1022-1003 of insert |
| MB21F11 | GCA CTG CAG AGG TGA TCA GC | 810-791 of insert |
| MB21F12 | AGG CCA GCT ACC TAC TGT CC | 197-178 of insert |
| MB21F13 | ACC TGT CTG GGC TGG AAT GG | 443-422 of insert |
| MB21F14 | GAT AGT CGA CCG CAC GTT GC | 570-551 of insert |

## Appendix 2: Oligonucleotide Primers

### (C) Oligonucleotide primers used to sequence *M. bovis* insert DNA from subclone 56/2

* = Nucleotides within sequence shown in Figure 5.2. The ORF extends from base 361 to 1825.

NB: 'R' primer sequences hybridise to the complementary DNA strand.

| Sequencing Primer | Oligonucleotide Primer Sequence | Nucleotides To Which Primer Hybridises* |
|---|---|---|
| MB21R2 | GGA ACC ACA AGC TGT ACG CG | 776-795 |
| MB21R3 | TCA ACA CGC TGC TGG TGA GC | 965-984 |
| MB21R7 | CGC GGT ACA GGC CAA TCA GG | 924-943 |
| MB21R9 | GTG GTG TGC CCT TCT TAC CG | 1229-1248 |
| MB21R10 | AGC CAA GGT GAA GAT CGG CG | 1452-1471 |
| MB21R11 | GCG TCG CTG TAC TCC GAT CC | 1597-1616 |
| MB21F3 | TAT CGG TCA AGG TGA CGT GC | 1036-1017 |
| MB21F5 | GTT CGA AGG CCA ACC GCG CG | 1159-1140 |
| MB21F6 | CGT CCC GAT GTC GTT GAT GC | 1296-1277 |
| MB21F7 | CAG GTT CAA CCC GCC GAT CG | 1482-1463 |
| MB21F8s | GGA TCG GAG TAC AGC GAC GC | 1616-1597 |
| MB21F9 | TGG ACA ACG CCT GGT ACA CC | 1695-1676 |
| MB21F10 | CGC CAC CAT CAA CAT CGC | 1940-1923 |
| MB21Fs | GTG CCG CGT ACA GCT TGT GG | 799-780 |

## Appendix 3: Plasmid Insert DNA Sequences

Insert DNA from PhoA+ recombinants was sequenced using primers (pJ6 and pJ7) that hybridised to the flanking vector sequences. Ambiguous nucleotides: S = G or C; N = G, A, T or C; K = G or T. Deduced amino acid sequences of the longest ORF in frame with *phoA* are shown using the single letter code. The arrows indicate putative signal peptide cleavage sites. SD = putative Shine Delgarno sequence. The *Sau* 3A sites which join two distinct DNA fragments to form a single DNA insert are indicated.

### 3.1 Fully Sequenced DNA Inserts

**pE2**

ORF in frame with *phoA* is identical to the start of an ORF (bases 8,977 to 9,678) in *M. tuberculosis* cosmid SCY24G1 (EMBL accession: Z83858).

```
        10        20        30        40        50        60
GATCCAATCAACCTGCATTCGGCCTGCGGGCCCATTTGACAATCGAGGTAGTGAGCGCAA

        70        80        90       100       110       120
ATGAATGATGGAAAACGGGCGGTGACGTCCGCTGTTCTGGTGGTGCTAGGTGCCTGCCTG
 M  N  D  G  K  R  A  V  T  S  A  V  L  V  V  L  G  A  C  L

       130       140       150       160       170       180
GCGTTGTGGCTATCAGGATGTTCTTCGCCGAAACCTGATGCCGAGGAACAGGGTGTTCCC
 A  L  W  L  S  G ↑C  S  S  P  K  P  D  A  E  E  Q  G  V  P

       190       200       210      218
GTGAGCCCGACGGCGTCCGACCCCGCGCTCCTCGCCGA / GATC
 V  S  P  T  A  S  D  P  A  L  L  A  E / PhoA
```

**pE5**

Bases 1 to 351 are identical to bases 33,620 to 33,270 of *M. tuberculosis* cosmid SCY20G9 (EMBL accession: Z77162).

```
        10        20        30        40        50        60
GATCTGGGCGGCGTCAAAAACCCGGCCCATCGCCGGGGTCCTTGTTATTCCTCTCCTG

        70        80        90       100       110       120
TTGCTTATAGAACGCGCCAAGCGTGCCATGCCAGATAAGCATCATGTCCCGCATCGTCGT
                           M  P  D  K  H  H  V  P  H  R  R

       130       140       150       160       170       180
CATCGCAGATATCATCGGCGGCATCTGCGCGCGCATCTGCGGCAACAGTGTAATCAGAAT
 H  R  R  Y  H  R  R  H  L  R  A  H  L  R  Q  Q  C  N  Q  N

       190       200       210       220       230       240
ATCCATATCCTTGACCAGGTACTCGAGCTTTTCGCTCAGCTGGTCCACACTGTCAAACAT
 I  H  I  L  D  Q  V  L  E  L  F  A  Q  L  V  H  T  V  K  H

       250       260       270       280       290       300
ATCGAATATCGATCTTATCGACCAGCAGAGCGGAACGTCGAAACAATGTTTTTCCCAGTA
 I  E  Y  R  S  Y  R  P  A  E  R  N  V  E  T  M  F  F  P  V

       310       320       330       340       350 Sau3A 360
GAAGTAGCTGCGAATAGGCCGGAAGAAATCCTCGAAATCCGCGAGATGATCGACCGCCGC
 E  V  A  A  N  R  P  E  E  I  L  E  I  R  E  M  I  D  R  R

       370       380       390       400       410       420
CACATCGGTGGCCAGCAACAGCAGCATGTCCGGCAGGCAGAGGTCATCGACGGCCCGTTC
 H  I  G  G  Q  Q  Q  Q  H  V  R  Q  A  E  V  I  D  G  P  F

       430       440       450       460       470       480
CGGCAATCGTTCCAGCCGTCGCACCATGTCATAGGCGAAGAAACCGACCATGCCACCCGA
 R  Q  S  F  Q  P  S  H  H  V  I  G  E  E  T  D  H  A  T  R

       490       500      509
CAGCGGCGGAAGACCCGGCTCGGACTGAC / GATC
 Q  R  R  K  T  R  L  G  L  T / phoA
```

**pM21**

ORF in frame with *phoA* is similar to the start of an ORF (bases 28,586 to 29,995) in *M. leprae* cosmid B1756 (Genbank accession: U15180).

```
         10         20         30         40         50         60
GATCTTGGAGTCGCGGGCCCACTTCTCCAGCAGTGATTGCTCGGAATACCCGGTAGTTCC

         70         80         90        100        110        120
TGCCAATTCGACGGTTTTGCAGGTGACGTCGCTGGCCATCCGGCCCGCCTTGGCCTTGCT

        130        140        150        160        170        180
CATCGAGGCTTCTTTGGAGTTGGGGATGTTGTTGTCGGCCTGCCAGGCTGCGCGCAGGGA

        190        200        210        220        230        240
CAGTAGGTAGCTGGCCTCCCAGTCGGCCTCCATCCGCAGGAACTCGGCGGCCGCGGCGCT

        250        260        270        280        290        300
CTGGGTGTGTGAGGGCTTGTCGTAGGAGATCAACCTATCCCGGCAACGATGCTCGCCGTT
                           Sau 3A

        310        320        330        340        350        360
CGGTGGGCCTCGGTCGCTCGCGGGTTGAGTGGATAGTGTGCCGGGAGTAGCTGGACCTGA

        370        380        390        400        410        420
CTGGACATGAAACGATGGCGCTGAAAAAGGGGGCGGAGGAGAATGAGAACCGATGACTA

        430        440        450        460        470        480
GCCCATTCCAGCCCAGACAGGTTCCCGGTTCAACACCCGCCGCCGCAGGTGCGGGTCGAC

        490        500        510        520        530        540
GTGGTGTGCCCGCATTGCCCACCCCGCCGAAAGGTTGGCCAGTCGGGTCGTATCCCACCT

        550        560        570        580        590        600
ATGCCGAGGCGCAACGTGCGGTCGACTATCTATCCGAGCAGCAGTTCCCGGTCCAGCAGG

        610        620        630        640        650        660
TGACCATCGTTGGCGTGGACCTCATGCAGGTTGAACGGGTCACAGGCCGGCTGACCTGGC

        670        680        690        700        710        720
CCAAAGTGCTTGGTGGCGGCGTGCTGAGTGGCGCCTGGCTGGGCCTGTTCATCGGGTTGG

        730        740        750        760        770        780
TGCTCGGGTTCTTCAGTCCCAATCCATGGTCCGCGCTGGTTACCGGCCTGGTGGCCGGGG

        790        800        810        820        830        840
TGTTCTTCGGGCTGATCACCTCTGCAGTGCCGTACGCAATGGCTCGCGGCACAAGGATT

        850        860        870        880        890        900
TCAGCTCGACCATGCAACTGGTTGCCGGTCGCTACGACGTACTTTGTGATCCGCAAAATG
```

**pM21 cont...**

```
       910       920       930       940       950       960
CGGAAAAGGCACGGGATCTGCTGGCGCGTCTGGCGATCTGAAGCCCGGACGAGAGGCAAA
                                                   SD

 ORF   970       980       990      1000      1010      1020
TGTGGTCATGAGTCGCGGGCGGATACCGAGGCTGGGCGCTGCCGTACTGGTGGCGTTGAC
  V  V  M  S  R  G  R  I  P  R  L  G  A  A  V  L  V  A  L  T

      1030      1040      1050      1060      1070      1080
GACCGCGGCGGCGGCGTGCGGGGCCGATAGCCAGGGGCTGGTGGTCAGCTTCTACACACC
  T  A  A  A  A ↑C  G  A  D  S  Q  G  L  V  V  S  F  Y  T  P

      1090      1100      1110      1120      1130      1140
GGCCACCGACGGCGCGACGTTCACCGCAATTGCCCAACGCTGCAACCAACAGTTCGGCGG
  A  T  D  G  A  T  F  T  A  I  A  Q  R  C  N  Q  Q  F  G  G

      1150      1160      1170      1180      1190      1200
CCGGTTCACCATTGCGCAGGTCAGCTTGCCCAGGTCCCCCAATGAGCAACGGTTACAGCT
  R  F  T  I  A  Q  V  S  L  P  R  S  P  N  E  Q  R  L  Q  L

      1210      1220      1230      1240      1250      1260
GGCCCGACGGTTGACCGGTAACGACCGCACCCTGGACGTCATGGCGCTGGATGTGGTGTG
  A  R  R  L  T  G  N  D  R  T  L  D  V  M  A  L  D  V  V  W

      1270      1280      1290      1300      1310      1320
GACGGCGGAGTTCGCCGAAGCGGGGTGGGCGCTGCCGCTGTCGGACGACCCAGCGGGGCT
  T  A  E  F  A  E  A  G  W  A  L  P  L  S  D  D  P  A  G  L

      1330      1340      1350      1360      1370      1380
GGCCGAGAACGACGCCGTCGCCGATACCCTGCCAGGCCCGCTTGCGACGGCCGGCTGGAA
  A  E  N  D  A  V  A  D  T  L  P  G  P  L  A  T  A  G  W  N

      1390      1400      1410      1420      1430      1440
CCACAAGCTGTACGCGGCACCCGTCACCACTAATACTCAATTGCTTTGGTACCGACCAGA
  H  K  L  Y  A  A  P  V  T  T  N  T  Q  L  L  W  Y  R  P  D

      1450      1460      1470      1480      1490      1500
TTTGGTAAATAGCCCGCCAACGGATTGGAATGCCATGATCGCTGAGGCGGCCCGGCTGCA
  L  V  N  S  P  P  T  D  W  N  A  M  I  A  E  A  A  R  L  H

      1510      1520
CGCGGCGGGCGAGCCTAGCTG / GATC
  A  A  G  E  P  S  W / PhoA
```

### 3.2 Entire Insert Sequenced On One DNA Strand Only

**pE14**

Entire pE14 DNA insert is identical to a bases 72 to 401 of pE9 insert.

```
        10        20        30        40        50        60
GATCTAGCTGAAGACCAAACCGGCACAGCAGACATTGCCATACGCGACAACAGCCGTCAT

        70        80        90       100       110       120
CAACCGAAAGGAGCAAAGAACAAACAGATGCATCCAATGATACCAGCGGAGTATATCTCC
                           M  H  P  M  I  P  A  E  Y  I  S

       130       140       150       160       170       180
AACATAATATATGAAGGCCCGGGCGCTGACTCATTGTTTTTCGCCTCCGGGCAATTGCGA
 N  I  I  Y  E  G  P  G  A  D  S  L  F  F  A  S  G  Q  L  R

       190       200       210       220       230       240
GAATTGGCTTACTCAGTTGAAACGACGGCTGAGTCGCTCGAGGACGAGCTCGACGAGCTG
 E  L  A  Y  S  V  E  T  T  A  E  S  L  E  D  E  L  D  E  L

       250       260       270       280       290       300
GATGAGAACTGGAAAGGTAGTTCGTCGGACTTGTTGGCCGACGCGGTTGAGCGGTATCTC
 D  E  N  W  K  G  S  S  S  D  L  L  A  D  A  V  E  R  Y  L

       310       320       330       340    347
CAATGGCTGTCTAAACACTCCAGTCAGCTTAAGCATGCCGCCTGGGT / GATC
 Q  W  L  S  K  H  S  S  Q  L  K  H  A  A  W  V / PhoA
```

## 3.3 Partially Sequenced DNA Inserts

**pEM1**

Identical to bases 12,910 to 13,202 of *M. tuberculosis* cosmid cY13c5 (incomplete sequence in *M. tuberculosis* database).

```
        10        20        30        40        50        60
GCGATCGGTGTGCTAGTCGGTGTGCAGGGCCACTCCGCGGCACCGCAGCGGGCGGCCGTG
 A  I  G  V  L  V  G  V  Q  G  H  S  A  A  P  Q  R  A  A  V

        70        80        90       100       110       120
TCGGCGCTGCCGATGGCCCAGGTCGGCACGCAGCTGTTGGCGTCCACGGTGTCGATCAGC
 S  A  L  P  M  A  Q  V  G  T  Q  L  L  A  S  T  V  S  I  S

       130       140       150       160       170       180
GGCGAGCCTTGGGGGACGTTCATCAACCTGCGGTGCGTCTGCCTGGCGCCGCCGTATGCT
 G  E  P  W  G  T  F  I  N  L  R  C  V  C  L  A  P  P  Y  A

       190       200       210       220       230       240
TCCCACGACACGCTGGCCATGGTTGTGGTGGGTCGTGACGGCAGCCAGACACGGCTGGCG
 S  H  D  T  L  A  M  V  V  V  G  R  D  G  S  Q  T  R  L  A

       250       260       270       280       290       300
ACTTGGTTGGCCGAACCCGGTCACACCGCGACACCCGCCGGCAGCATTTCGACACCGGTT
 T  W  L  A  E  P  G  H  T  A  T  P  A  G  S  I  S  T  P  V

  305
GACCA / GATC
 D  Q / PhoA
```

**pEM4**

ORF in frame with *phoA* is identical to the start of an ORF (bases 36,737 to 36,393) in *M. tuberculosis* cosmid SCY25D10 (EMBL accession: Z95558).

```
         10        20        30        40        50        60
GCCTTCGCCGGCATGCCCGCACTCCTGGACATCCGCGAAGAGCCCAACGGGCTGCAGTCG

         70        80        90       100       110       120
ATCGGTGGCTGGCTGCTCTCGGCCCACCTGGGCTGACGGCGCACCCAAACGACGCGTATA

        130       140       150       160       170       180
GTCCGGCTAATGAAGGGAACAAAGCTGGCTGTTGTCGTCGGCATGACGGTGGCTGCCGTT
          M  K  G  T  K  L  A  V  V  V  G  M  T  V  A  A  V

        190       200       210       220       230       240
AGTTTGGCAGCGCCGGCGCAGGCCGACGACTACGACGCCCCCTTCAACAACACGATCCAT
 S  L  A  A  P  A  Q  A  D  D  Y  D  A  P  F  N  N  T  I  H
                  ↑     ↑

     248
CGCTTCGG / GATC
 R  F  G / PhoA
```

**pE8**

No significant homology to known sequences.

```
         10        20        30        40        50        60
CACCGAAATCAGCAGATAGCAGCTATGATTAGCACCAGAATCCGACTAGCCATTATGTCC

         70        80        90       100       110       120
AGTACGACCAAACAAGTACATATACGAACCGCCAAATTTCGATTCAGCACAAAGCAAACC

       130       139
ATTACAGACCAAGTGCATG / GATC
```

**pE9**

The start of the ORF in frame with *phoA* is identical to the start of an ORF (bases 3,404 to 3,934) in *M. tuberculosis* cosmid Y78 (EMBL accession: Z77165).

```
        10        20        30        40        50        60
ACGACCGAGGCGTGTCGGCTACGCCGAGGCCCAAAACAGCAACAACCTTGCACTGATCTA

        70        80        90       100       110       120
GCTGAAGACCAAACCGGCACAGCAGACATTGCCATACGCGACAACAGCCGTCATCAACCG

       130       140       150       160       170       180
AAAGGAGCAAAGAACAAACAGATGCATCCAATGATACCAGCGGAGTATATCTCCAACATA
                      M  H  P  M  I  P  A  E  Y  I  S  N  I

       190       200       210       220       230       240
ATATATGAAGGCCCGGGCGCTGACTCATTGTTTTTCGCCTCCGGGCAATTGCGAGAATTG
 I  Y  E  G  P  G  A  D  S  L  F  F  A  S  G  Q  L  R  E  L

       250       260       270       280       290       300
GCTTACTCAGTTGAAACGACGGCTGAGTCGCTCGAGGACGAGCTCGACGAGCTGGATGAG
 A  Y  S  V  E  T  T  A  E  S  L  E  D  E  L  D  E  L  D  E

       310       320       330       340       350       360
AACTGGAAAGGTAGTTCGTCGGACTTGTTGGCCGACGCGGTTGAGCGGTATCTCCAATGG
 N  W  K  G  S  S  S  D  L  L  A  D  A  V  E  R  Y  L  Q  W

       370       380       390       400       410       420
CTGTCTAAACACTCCAGTCAGCTTAAGCATGCCGCCTGGGTGATCAACGGCCTCGCGAAC
 L  S  K  H  S  S  Q  L  K  H  A  A  W  V  I  N  G  L  A  N

       430       440       450       460
GCCTATAACGACACACGTCGGAAGGTGGTACCCCCGGAGGA / GATC
 A  Y  N  D  T  R  R  K  V  V  P  P  E  E / PhoA
```

**pM2**

Similar to *M. tuberculosis htrA* gene in cosmid cY161 (Genbank accession: U15180, coding sequence 21,036 to 22,637).

```
        10        20        30        40        50        60
GCTCTCAACCGTCCAGTGTCGACGACCGGCCAGGCCGGCAACCAGAACACCGTGCTGGAC
 A  L  N  R  P  V  S  T  T  G  Q  A  G  N  Q  N  T  V  L  D

        70        80        90       100       110       120
GCCATTCAGACCGACGCCGCGATCAACCCCGGTAACTCCGGNGGCGCGCTGGTGAACATG
 A  I  Q  T  D  A  A  I  N  P  G  N  S  G  G  A  L  V  N  M

       130       140       150       160       170       180
AACGCTCAACTCGTCGGAGTCAACTCGGCCATTGCCACGCTGGGCGCGGACTCAGCCGAT
 N  A  Q  L  V  G  V  N  S  A  I  A  T  L  G  A  D  S  A  D

       190       200       210       220       230       240
GCGCAGAGCGGCTCGATCGGTCTCGGTTTTGCGATTCCAGTCGACCAGGCCAAGCGCATC
A  Q  S  G  S  I  G  L  G  F  A  I  P  V  D  Q  A  K  R  I

       250       260       270       280       290       300
GCCGACGAGTTGATCAGCACCGGCAAGGCGTNACATGCCTCCCTGGGTGTGCAGGTGACC
 A  D  E  L  I  S  T  G  K  A  X  H  A  S  L  G  V  Q  V  T

       310       320   326
AATGACAAAGACACSCCGGGCKCCAA / GATC
 N  D  K  D  T  P  G  X  K / PhoA
```

**pM3**

ORF in frame with *phoA* is identical to the start of an ORF (bases 41,006 to 41,586) within *M. tuberculosis* cosmid SCY13E12 (EMBL accession: Z95390).

```
        10        20        30        40        50        60
CCCGCTCGCGAGCAGACGCAGAATCGCCCATTTCGGCACGAAATTGGGCGATTCTGCGTC

        70        80        90       100       110       120
TGCTCGCACCCTGGAAGCTGGTGCGGCTGCCCAAAGGCTGTGATACTCGATGGAGCGCGA

       130       140       150       160       170       180
AGGCCCGAAGGAGGGCATGTGAACATCCGTTGCGGACTGGCCGCTGGGGCCGTCATCTGC
                   V  N  I  R  C  G  L  A  A  G  A  V  I  C

       190       200       210       220       230       240
TCGGCCGTCGCACTGGGAATTGCGCTGCACTCCGGTGACCCGGCGCGTGCGCTCGGACCG
 S  A  V  A  L  G  I  A  L  H  S  G  D  P  A  R  A  L  G  P

       250       260       270       280       290       300
CCGCCGGATGGCAGTTACTCCTTCAACCAGGCCGGAGTGTCCGGGGTGACGTGGACGATT
 P  P  D  G  S  Y  S  F  N  Q  A  G  V  S  G  V  T  W  T  I

       310       320       330       340       350       360
ACCGCGCTGTGCGATCAGCCGTCGGGAACCCGTAACATGAACGACTATTCTGACCCCATC
 T  A  L  C  D  Q  P  S  G  T  R  N  M  N  D  Y  S  D  P  I

       370       380       390       400       410
GTTTGGGCGTTCAACTGCGCTCTCAACGTGGTGAGTACGACGCCCCAACA / GATC
 V  W  A  F  N  C  A  L  N  V  V  S  T  T  P  Q  Q / PhoA
```

**pM4**

Bases 1 to 60 are identical to bases 26,710 to 26,585 of *M. tuberculosis* cosmid SCY21B4 (EMBL accession: Z80108) Bases 57 to 212 are identical to bases 10,641 to 10,700 of *M. tuberculosis* cosmid Y253 (EMBL accession: Z81368).

```
        10        20        30        40        50  Sau 3A 60
CTCCGCCCAACCCCGCACACCCCGGCAAGCGCCCGCGTTGATGCCGTCGGCAGGCGGATC
 T  H  S  D  R  Q  V  R  R  S  L  R  P  T  P  H  T  P  A  S

        70        80        90       100       110       120
CTGGCGGGGTTGTCGGCGGCCATCCCGCCCAACGCCAGCCTGCTCGCGGCCATCCCGCCC
 A  R  V  D  A  V  G  R  R  I  L  A  G  L  S  A  A  I  P  P

       130       140       150       160       170       180
AACGCCAGCCTGCTCGAGCGCGCGGCCGCAATTGGAGCCCGGTACCAGCAGGCGTTCGCG
 N  A  S  L  L  E  R  A  A  A  I  G  A  R  Y  Q  Q  A  F  A

       190       200        212
CTGATGTACGGCGAGATATTCACCATCACCGC / GATC
 L  M  Y  G  E  I  F  T  I  T  A / PhoA
```

## Appendix 4: Research Objectives For Control Of Bovine Tuberculosis In New Zealand

In 1991 the Possum and Bovine Tuberculosis Control National Science Strategy Committee (NSSC) was established to identify and co-ordinate national priorities for possum and tuberculosis research in New Zealand (Wright, 1995). Research objectives were divided into three phases based on the expected time required for adequate information to become available (Livingstone, 1992). Research programmes have been developed to address the specific objectives set for each phase of research.

The objective of short term research (five years) is to identify effective methods to prevent the spread of bovine tuberculosis into economically important tuberculosis-free areas of New Zealand (Livingstone, 1992). Many short term research programmes have a strong epidemiological focus and relevant aspects of possum ecology and possum/cattle interactions have also been identified as areas worthy of further investigation (Livingstone, 1992). Another priority for short term research is to develop new environmentally acceptable possum toxins, attractants, and bait presentation methods to increase the efficiency and effectiveness of possum control operations (Wright, 1995). Research on methods for monitoring the effectiveness of control operations is also necessary (Wright, 1995).

The goal of the medium term research is to find the means to enable animal products from endemic areas to compete internationally (Livingstone, 1992). Outcomes of medium term research projects are expected within ten years (Wright, 1995). The major emphasis of medium term research is to develop a cost-effective, internationally acceptable vaccine for cattle and deer, and perhaps possums (Livingstone, 1992). Vaccine-based research is divided into two important areas; 1) the reassessment of the efficacy of the *M. bovis* BCG vaccine and 2) the development of a subunit-based vaccine against bovine tuberculosis. In addition, research on the pathogenesis and diagnosis of bovine tuberculosis is also a

priority (Wright, 1995). Certain epidemiological projects also fit into the time frame for medium term research (Wright, 1995).

Long term research is aimed at identifying a cost-effective, humane, environmentally safe method of eradicating tuberculosis from possums, preferably by eradicating the possum population (Livingstone, 1992). Research into the scientific feasibility of biological control of possums, the type of control most likely to be successful, and biological control safety issues are of highest priority (Wright, 1995). The results of such research are expected within fifteen years, although the possum problem in transmission of bovine tuberculosis is unlikely to be solved in the next twenty years (Wright, 1995).

## Appendix 5: Sequence Submission To GenBank

```
************************************************************************************************

LOCUS       XXXXX       2237 bp    DNA             BCT       05-OCT-1997
DEFINITION  Mycobacterium bovis.
ACCESSION   AF028830
KEYWORDS
SOURCE      Mycobacterium bovis.
  ORGANISM  Mycobacterium bovis
            Eubacteria; Firmicutes; Actinomycetes; Mycobacteria;
            Mycobacteriaceae; Mycobacterium.
REFERENCE   1  (bases 1 to 2237)
  AUTHORS   Borich,S.M., Gormley,E.P. and Murray,A.
  TITLE     Identification of a Mycobacterium bovis gene coding for a putative
            maltose-binding protein (MalE)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2237)
  AUTHORS   Borich,S.M., Gormley,E.P. and Murray,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-OCT-1997) Department of Veterinary Pathology and
            Public Health, Massey University, Private Bag, Palmerston North
            5301, New Zealand
FEATURES             Location/Qualifiers
     source          1..2237
                     /organism="Mycobacterium bovis"
     CDS             695..2101
                     /gene="malE"
                     /note="putative maltose-binding protein"
                     /codon_start=1
                     /product="MalE "
                     /translation="VVMSRGRIPRLGAAVLVALTTAAAACGADSQGLVVSFYTPATDG
                     ATFTAIAQRCNQQFGGRFTIAQVSLPRSPNEQRLQLARRLTGNDRTLDVMALDVVWTA
                     EFAEAGWALPLSDDPAGLAENDAVADTLPGPLATAGWNHKLYAAPVTTNTQLLWYRPD
                     LVNSPPTDWNAMIAEAARLHAAGEPSWIAVQANQGEGLVVWFNTLLVSAGGSVLSEDG
                     RHVTLTDTPAHRAATVSALQILKSVATTPGADPSITRTEEGSARLAFEQGKAALEVNW
                     PFVFASMLENAVKGGVPFLPLNRIPQLAGSINDIGTFTPSDEQFRIAYDASQQVFGFA
                     PYPAVAPGQPAKVTIGGLNLAVAKTTRHRAEAFEAVRCLRDQHNQRYVSLEGGLPAVR
                     ASLYSDPQFQAKYPMHAIIRQQLTDAAVRPATPVYQALSIRLAAVLSPITEIDPESTA
                     DELAAQAQKAIDGMGLLP"
     gene            695..2101
                     /gene="malE"
BASE COUNT      387 a    699 c    755 g    396 t
ORIGIN
        1 gatcaaccta tcccggcaac gatgctcgcc gttcggtggg cctcggtcgc tcgcgggttg
       61 agtggatagt gtgccgggag tagctggacc tgactggaca tgaaacgatg gcgctgaaaa
      121 agggggcgg aggagaatga gaaccgatga ctagcccatt ccagcccaga caggttcccg
      181 gttcaacacc cgccgccgca ggtgcgggtc gacgtggtgt gcccgcattg cccaccccgc
      241 cgaaaggttg gccagtcggg tcgtatccca cctatgccga ggcgcaacgt gcggtcgact
      301 atctatccga gcagcagttc ccggtccagc aggtgaccat cgttggcgtg gacctcatgc
      361 aggttgaacg ggtcacaggc cggctgacct ggcccaaagt gcttggtggc ggcgtgctga
      421 gtggcgcctg gctgggcctg ttcatcgggt tggtgctcgg gttcttcagt cccaatccat
      481 ggtccgcgct ggttaccggc ctggtggccg gggtgttctt cgggctgatc acctctgcag
      541 tgccgtacgc aatggctcgc ggcacaaggg atttcagctc gaccatgcaa ctggttgccg
      601 gtcgctacga cgtactttgt gatccgcaaa atgcggaaaa ggcacgggat ctgctggcgc
      661 gtctggcgat ctgaagcccg gacgagaggc aaatgtggtc atgagtcgcg ggcggatacc
      721 gaggctgggc gctgccgtac tggtggcgtt gacgaccgcg gcggcggcgt gcggggccga
      781 tagccagggg ctggtggtca gcttctacac accggccacc gacggcgcga cgttcaccgc
      841 aattgcccaa cgctgcaacc aacagttcgg cggccggttc accattgcgc aggtcagctt
      901 gcccaggtcc cccaatgagc aacggttaca gctggcccga cggttgaccg gtaacgaccg
      961 caccctggac gtcatggcgc tggatgtggt gtggacggcg gagttcgccg aagcggggtg
     1021 ggcgctgccg ctgtcggacg acccagcggg gctggccgag aacgacgccg tcgccgatac
     1081 cctgccaggc ccgcttgcga cggccggctg gaaccacaag ctgtacgcgg cacccgtcac
     1141 cactaatact caattgcttt ggtaccgacc agatttggta aatagcccgc caacggattg
     1201 gaatgccatg atcgctgagg cggcccggct gcacgcggcg ggcgagccta gctggatcgc
     1261 ggtacaggcc aatcagggcg agggcttagt ggtgtggttc aacacgctgc tggtgagcgc
     1321 tggtggatcg gtgctctccg aggacggccg gcacgtcacc ttgaccgata ctcccgcaca
     1381 ccgagcggct acggtcagcg cgctacagat cctcaaatcg gtggctacca cgcccggcgc
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1441 cgacccctcg atcacccgca ccgaagaggg cagcgcgcgg ttggccttcg aacagggcaa
1501 ggccgcgctc gaggtcaatt ggccgttcgt gtttgcgtcc atgctcgaga acgcggtgaa
1561 gggtggtgtg cccttcttac cgcttaaccg gattccgcag ttggccggca gcatcaacga
1621 catcgggacg ttcacgccca gcgacgagca gttccgcatc gcgtatgacg ccagccagca
1681 ggtgttcggt ttcgcgccct atccggctgt agcgcccggc cagccagcca aggtgacgat
1741 cggcgggttg aacctggcgg tggccaagac gacccgccat cgagcggagg cattcgaagc
1801 ggtgcgttgt ctgcgtgacc agcacaatca gaggtacgtc tcgctcgagg ggggtctgcc
1861 cgcggtgcgg gcgtcgctgt actccgatcc gcaattccag gcgaagtatc cgatgcacgc
1921 cattattcgg cagcaactca ccgatgccgc ggtgcggccg gcgacgccgg tgtaccaggc
1981 gttgtccatc cggctcgcgg cggtgctgag cccgatcacc gagatcgacc cggagtccac
2041 ggccgacgaa cttgccgcgc aggcgcagaa agccatcgac ggcatgggcc tgctcccgtg
2101 acctccgttg aacagcggac cgccaccgcg gtcttttccc gtaccgggag ccgcatggcc
2161 gaacggcgac tggcgttcat gctggtcgca cccgccgcga tgttgatggt ggcggtgacg
2221 gcctatccca tcggtta
//
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