CLONING AND EXPRESSION OF
MYCOBACTERIAL GENES IN ESCHERICHIA COLI

Thesis submitted to the University of Surrey
for the degree of Doctor of Philosophy
in the Department of Microbiology

by

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The ability of *Escherichia coli* to use the expression signals of mycobacterial genes was tested by inserting fragments of *M. bovis* BCG DNA into the *E. coli* promoter-probe plasmid pKK232-8. Comparison with the promoter activity achieved following insertion of restriction fragments of the *E. coli* host into pKK232-8 revealed that a significant proportion of *M. bovis* BCG promoters were functional in *E. coli*. These results confirmed the suitability of *E. coli* as a host for the cloning and expression of mycobacterial genes.

Using a variety of *E. coli* cloning vectors (pBR322, pUC13, EMBL4 and gtII), *M. bovis* BCG and *M. leprae* DNA gene libraries were prepared. Recombinant *M. bovis* BCG clones were screened with rabbit antiserum and clones expressing *M. bovis* BCG antigens were identified. A pBR322/*M. bovis* BCG clone, expressing a 65KD molecule, was isolated and this antigen was shown to be cross-reactive with a 65KD *M. leprae* antigen. Recombinant gtII clones, expressing antigenic *M. bovis* BCG molecules, were also detected and a partial DNA sequence was determined for one of these molecules. Moreover, recombinant gtII clones expressing (i) an 85KD biotinylated *M. leprae* molecule and (ii) an 85KD biotinylated *M. bovis* BCG molecule were also detected.

In an attempt to test the feasibility of diagnosing leprosy by the presence of antibodies to specific antigens, antisera samples from leprosy patients and their contacts were screened for antibodies to mycobacterial antigens. Although only a small number of antisera were tested, a number of candidate antigens were identified.
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<td>294</td>
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</table>
Section 1  The Genus Mycobacterium

The genus *Mycobacterium* was first named in 1896, by Lehmann and Neumann, and at that time contained only two members: *Mycobacterium leprae*, the causative organism of leprosy (described by Armauer Hansen in 1874), and *Mycobacterium tuberculosis*, the causative organism of tuberculosis (described and cultured by Robert Koch in 1882). The name *Mycobacterium* means "fungus-bacterium", and is derived from a description of the characteristic fungus-like pellicle produced by *M. tuberculosis*, when grown on liquid media (Collins et al., 1985).

Since its first description, over 90 years ago, a number of new species have been ascribed to the genus *Mycobacterium*, ranging from pathogens of man and animals (eg *M. bovis*, *M. leprae*, *M. paratuberculosis* etc.) to environmental saprophytes that do not normally cause disease (eg *M. phlei*, *M. smegmatis*, *M. nonchromogenicum* etc.). In 1980, Skerman et al. published a list of "Approved Bacterial Names" and attributed forty-one species to the genus *Mycobacterium* (Table 1). Ratledge and Stanford (1982) noted however, that only sixteen of these forty-one species had survived from an earlier published list of 128 mycobacterial species (Index Bergeyana, 1966). These authors further noted that the "Approved List" had come in for criticism from a number of sources for (i) listing some organisms as
species, rather than as sub-species and (ii) for not listing some other validly recognised species.

The most distinctive property of the Mycobacteriaceae is their ability to stain red following treatment with the Ziehl-Neelsen stain, then resist decolourisation with acid alcohols. Despite recognition of the property of acid-fastness over 100 years ago, this property is still not fully understood. Workers have suggested that mycolic acids (constituents of mycobacterial cell walls), comprise the acid-fast material of mycobacterial cells, reporting isolated mycolic acids showing acid-fastness (Stodola et al., 1938). However, Kanai (1962) noted that acid-fastness was dependent upon intact mycobacterial cells, with acid-fastness completely lost if the cells are broken, a fact not in keeping with a role for mycolic acids; however other workers have also noted that the removal of the outer walls of mycobacteria leaves intact cells which are non-acid fast, thus reaffirming a role for mycolic acids in acid-fastness. Youmans and Youmans (1966 a;b) have also suggested a possible role for mycobacterial RNA in the acid-fast staining process, following their isolation of a mycobacterial RNA-protein complex from mycobacterial cells, which when stained by the Ziehl-Neelsen method became red and moreover did not decolourise with acid-alcohol. Consequently, Barksdale and Kim (1977) considered the property of acid-fastness to be dependent upon two reactions: (1) the uptake of carbol fuchsin into the cell's interior and the binding of the carbol fuchsin to the mycolic acid residues of the outer cell wall and (2) the subsequent trapping of the carbol fuchsin within the cell by the formation of stable, fuchsin-mycolate complexes which are strongly acid-
fast and furthermore are unable to penetrate the cell membrane to leave the cell.

Three further distinctive properties have been described for mycobacteria, allowing organisms to be classified within this genus. First, mycobacteria contain an extremely lipophilic cell wall, with c.60% of the wall composed of lipids (Draper, 1982). This high lipid content of mycobacterial cell walls is consequently responsible for their extreme hydrophobicity and probably also contributes to their slow growth, by hindering the uptake of nutrients into the organism. Secondly, a high proportion of mycobacterial cell wall lipids exist in the form of mycolic acids (Ratledge, 1982), which are high molecular weight, branched chain, fatty acids. Although related bacteria also contain mycolic acids, those of mycobacteria are uniquely composed of 60-90 carbon atoms and furthermore mycobacteria mycolic acid patterns are species-specific (Minnikin et al., 1984), encouraging these authors to propose that mycolic acid analysis be employed in the classification of mycobacterial species. Thirdly, mycobacterial genomes have a characteristically high G+C content, observed to be in the range of 61-71% by a number of workers (Baess and Mansa, 1978; Baess, 1984; and Imaeda, 1985). Table 2 has been compiled from the work of the above authors and lists the %G+C values for twenty-three species of mycobacteria, together with details of their genome sizes.

Since the work of Hansen and Koch, determining the causitive organisms of leprosy and tuberculosis respectively, work with the genus *Mycobacterium* has principally revolved around the role of mycobacteria
as pathogens of man and animals. Clinically, mycobacteria have been considered as three groups: (1) pathogens of man and animals; (2) species causing opportunist infections and (3) species that never or only very rarely cause infection. Table 1 was further compiled from the work of Collins et al. (1985); Grange (1980); Goodfellow and Wayne (1982); Wayne (1985); Collins et al, (1984); Chadwick (1982) and Barksdale and Kim (1977) and describes the clinical classification of the forty-one recognised species of mycobacteria.
<table>
<thead>
<tr>
<th>Species</th>
<th>Clinical Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. africanum</td>
<td>human pathogen</td>
</tr>
<tr>
<td>M. asiaticum</td>
<td>rarely pathogenic</td>
</tr>
<tr>
<td>M. aurum</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. avium</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. bovis</td>
<td>human pathogen, animal pathogen</td>
</tr>
<tr>
<td>M. chelonel</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. chitae</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. duvalii</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. farcinogenes</td>
<td>animal pathogen</td>
</tr>
<tr>
<td>M. flavescens</td>
<td>rarely pathogenic</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. gadium</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. gastri</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. gilvum</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>rarely pathogenic</td>
</tr>
<tr>
<td>M. haemophilum</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. komossense</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. leprae</td>
<td>human pathogen</td>
</tr>
<tr>
<td>M. lepraemurium</td>
<td>animal pathogen</td>
</tr>
<tr>
<td>Species</td>
<td>Clinical Classification</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>M. malmense</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. marinum</td>
<td>human pathogen, animal pathogen</td>
</tr>
<tr>
<td>M. microti</td>
<td>animal pathogen</td>
</tr>
<tr>
<td>M. neoaurum</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. nonchromogenicum</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. parafortuitum</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. paratuberculosis</td>
<td>animal pathogen</td>
</tr>
<tr>
<td>M. phlei</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. senegalense</td>
<td>animal pathogen</td>
</tr>
<tr>
<td>M. simiae</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. terrae</td>
<td>rarely pathogenic</td>
</tr>
<tr>
<td>M. thermoresistibile</td>
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</tr>
<tr>
<td>M. triviale</td>
<td>rarely pathogenic</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>human pathogen, animal pathogen</td>
</tr>
<tr>
<td>M. ulcerans</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>M. vaccae</td>
<td>not normally pathogenic</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>opportunist pathogen</td>
</tr>
<tr>
<td>Mycobacterial strains</td>
<td>G+C Percentage</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><em>M. africanum</em> (TMC 5122)</td>
<td>62.7</td>
</tr>
<tr>
<td><em>M. asiaticum</em> (TMC 803)</td>
<td>63.1</td>
</tr>
<tr>
<td><em>M. avium</em> (ATCC 25291)</td>
<td>70.3</td>
</tr>
<tr>
<td><em>M. bovis</em> (ATCC 19210)</td>
<td>62.7</td>
</tr>
<tr>
<td><em>M. bovis BCG</em> (Glaxo) (TMC 1024)</td>
<td>62.7</td>
</tr>
<tr>
<td><em>M. fortuitum</em> (ATCC 6841)</td>
<td>66.0</td>
</tr>
<tr>
<td><em>M. gastri</em> (ATCC 25158)</td>
<td>66.6</td>
</tr>
<tr>
<td><em>M. gordonae</em> (ATCC 14470)</td>
<td>66.1</td>
</tr>
<tr>
<td><em>M. haemophilum</em> (TMC 804)</td>
<td>61.5</td>
</tr>
<tr>
<td><em>M. intracellularum</em> (ATCC 25169)</td>
<td>70.0</td>
</tr>
<tr>
<td><em>M. kansasii</em> (ATCC 12478)</td>
<td>67.5</td>
</tr>
<tr>
<td><em>M. leprae</em> (Hawaiian)</td>
<td>65.5</td>
</tr>
<tr>
<td><em>M. marinum</em> (ATCC 927)</td>
<td>66.5</td>
</tr>
<tr>
<td><em>M. microti</em> (TMC 1608)</td>
<td>62.3</td>
</tr>
<tr>
<td><em>M. nonchromogenicum</em> (TMC 1481)</td>
<td>64.7</td>
</tr>
<tr>
<td><em>M. parafortuitum</em> (ATCC 19686)</td>
<td>69.2</td>
</tr>
<tr>
<td><em>M. phlei</em> (ATCC 27086)</td>
<td>71.4</td>
</tr>
<tr>
<td><em>M. scrofulaceum</em> (ATCC 19073)</td>
<td>70.2</td>
</tr>
<tr>
<td><em>M. simiae</em> (ATCC 25275)</td>
<td>69.1</td>
</tr>
<tr>
<td><em>M. smegmatis</em> (ATCC 14468)</td>
<td>68.3</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> (TMC 102)</td>
<td>63.3</td>
</tr>
<tr>
<td><em>M. vaccae</em> (ATCC 15483)</td>
<td>70.2</td>
</tr>
<tr>
<td><em>M. xenopi</em> (SSC 989)</td>
<td>68.2</td>
</tr>
</tbody>
</table>
1.1 Techniques for the Classification of Mycobacterial Species and Strains

In order to classify species, a number of techniques have been developed which allow interspecies and intraspecies variation to be determined. These techniques include: biochemical tests; immunological analysis and DNA homology data.

(1) The first coherent strategy for classifying mycobacteria was developed by Gordon and her colleagues (1953, 1955) when they successfully achieved the delineation of fast growing mycobacterial species on the basis of a number of biochemical tests, stressing the importance of reaction patterns rather than results of single tests. As a consequence of this work and the pioneering work by Wayne (1967), in the development of biochemical tests for the classification of slow growing mycobacteria, cooperative studies were undertaken, under the auspices of the International Working Group on Mycobacterial Taxonomy (IWGNT), resulting in the identification of tests for the efficient classification of mycobacterial species (Goodfellow and Wayne, 1982). Biochemical tests allow the determination of a large number of characteristic properties for each individual bacterial strain, which can then be clustered into species according to their degree of similarity.

(2) Using the technique of immunodiffusion analysis, Stanford and Grange (1974) described the classification of over 1000 mycobacterial strains into twenty separate species. This technique
demonstrated the presence of four groups of mycobacterial antigens: group (i) antigens, common to all mycobacterial strains; group (ii) antigens, shared between all species of slow growers; group (iii) antigens, shared between all fast growing species and group (iv) antigens, unique to individual species (and it was these group (iv) antigens which allowed speciation to be carried out). Furthermore, these authors demonstrated that this technique was also capable of recognising subspecies.

(3) DNA hybridisation techniques have been used by a number of workers to study the relatedness of mycobacterial strains, as a means to achieve their classification. Baess (1979) demonstrated a high degree of homology between *M. tuberculosis* and both *M. bovis* and *M. bovis* BCG and proposed that the one species *M. tuberculosis* be reintroduced and Imaeda (1985) demonstrated that *M. africanum* also belongs to the *M. tuberculosis* complex on the basis of DNA homology. Baess (1979) also demonstrated that strains of *M. avium* and *M. intracellulare* can be clearly divided into two groups, one consisting of *M. avium* and *M. intracellulare* Davis and the second comprising the other *M. intracellulare* strains. By this technique, the *M. scrofulaceum* strain was determined to be definitely different from both *M. avium* and *M. intracellulare*. Baess (1982) has also used this technique to compare the relatedness among species of rapidly growing mycobacteria. More recently McFadden et al. (1987a) have used this technique to demonstrate homology between an unclassified mycobacterium species isolated from three patients with Crohn's disease and *M. paratuberculosis*, advocating a role for *M. paratuberculosis* in Crohn's disease.
The classification of mycobacterial species and strains has presented many problems in the past, however it is fully anticipated that by using the three techniques described above, workers should be able to readily assign newly isolated strains to defined species.
According to Collins et al. (1985), 50 to 100 million people worldwide are infected each year with the tubercle bacillus and of these, c. 10 to 20 million actually develop disease symptoms and 3 million subsequently die of the disease. Leprosy is also a chronic infectious disease of man and according to WHO figures (1979) there are an estimated 10-15 million sufferers, the majority living in developing countries. As a significant number of these sufferers (20-30%) also suffer from deformities, as a result of their infection, it is highly understandable why leprosy also represents a major health problem (Bloom and Godal, 1983).

Not unnaturally, *M. tuberculosis* and *M. leprae* have dominated studies on the genus *Mycobacterium*, however a number of environmental mycobacterial species are also capable of causing disease in man. In south-east England the most prevalent environmental pathogens are *M. xenopi, M. kansasii* and the *M. avium-intracellulare-scrofulaceum* group organisms (Yates et al., 1986). According to these same authors, environmental mycobacteria now account for over 5% of bacteriologically confirmed mycobacterial disease in south-east England.
2.1 Tuberculosis

The tubercle bacillus (\textit{Mycobacterium tuberculosis}) was isolated, cultured and described as the causative organism by Robert Koch in 1882, although the infectiousness of the disease had been previously demonstrated a number of years earlier, following experiments conducted by Jean Antoine Villemin between 1865 and 1868. With evidence from human remains to suggest its presence c.8000 years B.C., tuberculosis has long been regarded a disease of antiquity (Youmans, 1979).

Since its initial description by Lehmann and Neumann in 1896, a number of variants have been assigned to the species \textit{M.tuberculosis}; including human, African and bovine types. Two human variants have been described: the classical type originally described by Koch and a more recently described Asian variant (Dhayagude and Shah, cited by Collins et al., 1985), particularly prevalent in South India and among ethnic Asian patients living in other countries (Collins et al., 1985). The original African variant was isolated in West Africa and named \textit{M.africanum} by Castets et al. (cited by Collins et al., 1985), since then however, two African variants have been recognised: the African I variant and the African II variant, with both showing properties intermediate between the classical human and bovine types, which has also been accorded separate species status (Karlson and Lessel, 1970). Finally there is \textit{M.bovis} BCG, a laboratory prepared, attenuated variant of the bovine tubercle bacillus, which has been extensively used as a vaccine. This strain was attenuated from a virulent bovine tubercle strain, following 231 sub-cultures of this strain on a potato-glycerol-
bile medium by Calmette and Guérin. Despite the separate species status of a number of these variants, each can be assigned to the species *M. tuberculosis* on the basis of antigenic analysis (Stanford and Grange, 1974) and DNA hybridisation studies (Baess, 1979).

From a clinical point of view Collins et al. (1985) have suggested that it is sufficient to determine whether a mycobacterium is or is not *M. tuberculosis*, as further sub-division of this species into its variants does not provide information that affects the management of the patient. However these authors do consider sub-division into the respective variants necessary for providing information on the epidemiology of the disease in a population. According to Collins et al. (1985), clinical isolates of the tubercle bacillus from patients can be reliably sub-divided into the respective variants following the results of five simple tests: (i) sensitivity to 5mg/litre thiophene-2-carboxylic acid hydrazide (Harrington and Karlson, 1966), (ii) the nitratase test of Collins and Lyne (1984), (iii) the oxygen preference test of Marks (1972), (iv) the pyrazinamide sensitivity test (cited by Collins et al., 1985) and (v) sensitivity to 20mg/litre cycloserine (cited by Collins et al., 1985). Table 3 summarises the results expected for each of the *M. tuberculosis* variants, to each of the five tests.
### Table 3: Species and Variants of Tubercle Bacilli

<table>
<thead>
<tr>
<th>Variant</th>
<th>TCH sensitivity</th>
<th>Nitratase activity</th>
<th>Oxygen preference</th>
<th>Pyrazinamide sensitivity</th>
<th>Cycloserine sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>R</td>
<td>+</td>
<td>A</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Asian</td>
<td>S</td>
<td>+</td>
<td>A</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>African I</td>
<td>S</td>
<td>-</td>
<td>M</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>African II</td>
<td>S</td>
<td>+</td>
<td>M</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Bovine</td>
<td>S</td>
<td>-</td>
<td>M</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>BCG</td>
<td>S</td>
<td>-</td>
<td>A</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

(Notes: TCH = thiophene-2-carboxylic acid hydrazide; S = sensitive; R = resistant; A = aerobic and M = microaerophilic.)

In practice simply testing clinical isolates on medium containing TCH (5mg/litre) is sufficient to determine whether the organism is *M. tuberculosis* or a variant.
2.1.1 Case Detection and Diagnosis of Tuberculosis

Three main methods exist for detecting cases of tuberculosis within the community: (1) indiscriminate mass radiography; (2) tuberculin skin-testing and (3) direct microscopical examination of pathological materials for acid-fast bacilli.

(1) In 1944, when indiscriminate mass radiography was introduced into Britain there were 54313 reported cases of tuberculosis. When this service was abandoned in 1969, for being too expensive (case detection success rates could not justify the huge expense of the programme), only one case of tuberculosis was being detected for every 2000 examinations, the majority of tuberculosis cases were still being detected as a result of patients presenting symptoms of the disease to their own doctors (Grange, 1980).

(2) The tuberculin test involves skin-testing individuals with PPD (Purified Protein Derivative of M.tuberculosis) and a positive response is indicative of the individual having had contact with M.tuberculosis. Unfortunately the test does not distinguish between active and inactive disease, moreover the test can only be used on individuals not previously vaccinated with M.bovis BCG, as vaccination with this organism normally renders tuberculin-negative individuals tuberculin-positive.

(3) Microscopical analysis and culture of sputum and other specimens, including urine and cerebrospinal fluid, is probably the most
useful test for the diagnosis of tuberculosis. Sputum can be stained by the Ziehl-Neelsen procedure and acid-fast bacilli detected by direct microscopy. Unfortunately direct microscopy is rather insensitive requiring 10000 acid fast bacilli per ml of sputum to give a positive smear. Consequently, sputum samples are also cultured on a variety of media, allowing samples initially considered negative by microscopy to be re-evaluated. Culturing organisms from sputum also allows their identification and verification as tubercle bacilli; allows drug sensitivity tests to be carried out and moreover allows chemotherapy regimens to be described to combat the disease. A number of different culture media have been developed for growing the tubercle bacillus in particular and mycobacteria in general (Jenkins et al., 1982), the most popular of which appear to be the various modified versions of Lowenstein-Jensen medium. Jenkins et al. (1985) described the inoculation and incubation of four Lowenstein-Jensen slopes, allowing rapid identification of the tubercle bacillus. Growth of acid-fast bacilli within 3 to 7 days suggests the organism is not \textit{M. tuberculosis}, which requires 6 to 8 weeks incubation before growth is achieved on Lowenstein-Jensen slopes, particularly on first isolation.

Individuals presenting symptoms of the disease or with "suspicious" shadows on chest X-rays, may suggest tuberculosis infection but final diagnosis of the disease is dependent upon isolation of the causative organism and its subsequent identification following growth on artificial media.
2.1.2 The Infection

Tuberculosis is a chronic infectious disease and can be caused by any variant of the \textit{M. tuberculosis} complex (Table 3). The most frequently infected organ is the lung, but the disease does occur at other sites including the lymph nodes, kidney, reproductive system, intestine, bone, skin and the central nervous system. Tuberculosis is often divided into pulmonary and extrapulmonary types, with the second term only applied to organ involvement which is secondary to a pulmonary lesion. Non-pulmonary tuberculosis refers to disease which has not involved the lung.

Tuberculous infection may occur in two general types of individuals: (1) in individuals that have had no previous experience with the tubercle bacillus, i.e. they have never been infected or diseased and (2) in individuals who have had previous experience with the tubercle bacillus, i.e. they have previously been infected or have previously been diseased or have previously been vaccinated with \textit{M. bovis} BCG. When infection occurs in an individual with no previous record of tuberculosis infection, the disease is referred to as primary tuberculosis. Secondary tuberculosis occurs in individuals who have previously been infected and the disease may be the result of reinfection from an active tuberculous case or the result of reactivation of an old infection.

Infection with the tubercle bacillus generally involves inhalation of air-borne bacilli and results in primary infection of the
lung. The inhaled bacilli lodge within an alveolus and are rapidly ingested by alveolar phagocytes. Because of their high resistance to destruction, the tubercle bacilli can survive and replicate within these cells. In individuals unable to mount a cellular immune response to the infection, multiplication of the tubercle bacilli continues unhindered from the primary focus which results in the dissemination of virulent bacilli via the lymphatics and bloodstream and the infection of the lymph nodes and other tissues. The majority of individuals however, within a few days of acquiring a primary infection, mount a cellular immune response which confines the disease to the site of the initial focus (Fok et al., 1976) and stimulates the phagocytes to commence the destruction of the ingested tubercle bacilli.

Not all individuals with tubercle infections are infectious. Frequently the immune response mounted to combat the tubercle infection does not result in the complete killing of all the bacilli, allowing viable, virulent bacilli to lie dormant within the host. These individuals however, are not infectious; infectiousness is dependent upon the presence of an active lesion, from which viable, virulent bacilli are being released and expelled into the environment. As a rule, only individuals whose sputum samples contain sufficient bacilli to be detected microscopically, are considered infectious. If dormant tubercle bacilli are reactivated, by any of a number of stimuli, e.g. age, degenerative disease, immunosuppressive-therapy or a new tubercle infection, then that individual will once more become infectious.
2.1.3 Immune Responses to Tubercle Infection

Following infection with a pathogen, the two arms of the specific defence system (i.e. the humoral and the cellular immune systems) can be activated and used to combat the infection. The humoral immune response is mounted by a group of lymphocytes, known as B-lymphocytes or B-cells (these cells are classically defined by the presence of endogenously produced immunoglobulin (antibody) molecules, inserted into the surface membrane where they act as specific antigen receptors). B-cells are activated when they bind their specific antigen and activation results in their proliferation and transformation into (i) blast cells and then (ii) plasma cells, which secrete immunoglobulins. The cell-mediated immune response is dependent upon a group of lymphocytes, known as T-lymphocytes (or T-cells) and within this "family" of cells, different types of T-cells have different roles: the cytotoxic T-cells bind to target cells and kill them; helper T-cells cooperate with B-cells to induce antibody production and can also release lymphokine molecules (which help macrophages to kill intracellular organisms); suppressor T-cells specifically suppress the action of macrophages, B-cells and helper T-cells and delayed hypersensitivity T-cells are responsible for the delayed response to antigen (these cells have been sensitised to the antigen by a previous encounter), (Roitt et al., 1985). Protection against tuberculosis (and leprosy) is known to be dependent upon the cell-mediated immune response to the infection.
On the basis of clinical, morphological and immunological studies (techniques previously used by Ridley and Jopling (1966) to classify patients with leprosy) Lenzini et al. (1977) described a disease spectrum for tuberculosis, which consisted of four groups: 1. reactive (RR); 2. reactive intermediate (RI); 3. unreactive intermediate (UI) and 4. unreactive (UU). The reactive form (RR) is characterised by localised lesions with lymphocytes and epithelioid cells and by an early response to chemotherapy. Furthermore, this form also shows evidence of active cell-mediated immunity but shows little or no antibody response. The unreactive form of the disease (UU) is characterised by dissemination of the infection to a number of organs and a poor response to chemotherapy. Immunologically this form of the disease shows a lack of cell-mediated immunity but high levels of antibody. The two intermediate forms of the disease show characteristics of the neighbouring polar forms (i.e. RR and UU), with RI patients showing slightly more cellular immunity than UI patients. Accordingly (like leprosy), an individual's placement within the tuberculosis disease spectrum is dependent upon the degree of cellular immunity expressed following infection with the tubercle bacillus.

Following infection with tubercle bacilli, phagocytes initially ingest the bacilli, then play a further role as antigen-presenting cells (APC). T-cells will only respond to invading bacilli when antigens from these organisms have been properly processed and presented. Moreover, effective presentation can only occur when the foreign antigen is combined with class I or class II major histocompatibility (MHC) molecules (Bodmer and Bodmer, 1984). Thus, helper T-cells usually
require foreign antigen to be presented in conjunction with self-class II MHC molecules (Nixon et al., 1982) and cytotoxic T-cells usually require the presentation of foreign-antigen in conjunction with self-class I MHC molecules (Zinkernagel and Doherty, 1975). The need for simultaneous recognition of antigen and MHC products by the T-cells has led to the following two hypotheses: (1) the dual receptor hypothesis suggests that the MHC product and the antigen are recognised by separate receptor molecules on the T-cell surface and (2) the associative recognition hypothesis suggests that antigen associates with the MHC products and is recognised by a single T-cell receptor (this hypothesis has been modified to produce the altered self hypothesis, according to which the self-MHC molecule on the antigen-presenting cell is somehow modified by the presence of antigen and a single T-cell receptor subsequently recognises both antigen and altered self-MHC) (Roitt et al., 1985).

The binding of helper T-cells to an antigen plus class II MHC complex activates release of interleukin-1 (IL-1) from the antigen-presenting cell (Unanue et al., 1984) and this lymphokine molecule then stimulates helper T-cells to produce interleukin-2 (IL-2), which promotes T-cell proliferation. Once activated, T-cells release lymphokine molecules which mediate a number of biological effects (and it is at this stage that the two arms of the defence system diverge). The types of lymphokine molecules secreted are dependent upon the type of antigen presented and thus upon the responding helper T-cell: (i) antigen creating a predominantly T-cell dependent, B-cell response will stimulate the release of lymphokines which will activate antigen-bound
B-cells (leading to their transformation into antibody secreting cells) and (ii) antigen activating cellular immune responses stimulate macrophage and/or cytotoxic T-cell activating lymphokines.

In tuberculosis (and leprosy) the most important part of the cellular immune response is the effective activation of macrophages. Lymphokines have been shown to activate monocytes from the blood, which then report to infection sites and mature into macrophages, whereupon they commence phagocytosis of the invading bacilli (Volkman and Gowans, 1965a; b; Mackaness, 1969). A lymphokine molecule capable of preventing macrophages from leaving infection sites has also been described (Bloom and Bennett, 1966; David, 1966). An activated macrophage differs from an unstimulated macrophage in a variety of ways: it has a ruffled cell membrane (increasing its surface area); an increased ability to phagocytose foreign matter; an increased number of organelles (including the number of lysosomes) and increased bactericidal activity (Karnovsky and Lazdins, 1978).

Following phagocytosis by activated macrophages, the ingested tubercle bacilli lie within vacuoles (termed phagosomes) but before these bacilli can be destroyed, the phagosome must first fuse with one or more lysosomes to produce a phagolysosome. Following phagosome-lysosome fusion, the tubercle bacilli are usually killed by the action of a number of the many bactericidal substances contained within the lysosomes.
With protection from tuberculosis dependent upon the expression of an effective cell-mediated immune response (leading to the activation of macrophages and the destruction of the invading bacilli) a number of workers have examined tuberculosis patients and have succeeded in detecting defects in the cell-mediated immune responses of these individuals: Cruchaud et al. (1977) described reduced phagocytic and bactericidal capacities of macrophages from individuals with severe pulmonary tuberculosis; other workers (Al-Tawil et al., 1978; Skvor and Trnka, 1979) have reported the relative proportions of T-cells to be lower in tuberculosis patients than in controls (Malaviya et al., 1975; reported no significant differences in T-cell numbers between tuberculous patients and controls); Ellner (1978) has reported the presence of circulating suppressor T-cells in tuberculosis patients and Katz et al. (1979) have reported that untreated tuberculosis patients had increased numbers of suppressor T-cells and low numbers of helper T-cells. Thus any number of factors may account for a less than effective cell-mediated immune response to infection with tubercle bacilli, which may possibly result in the infection escalating to the full-blown disease state.
2.1.4 Control of Tuberculosis

Control of tuberculosis is dependent upon two factors: (1) decreasing the number of infectious individuals in the community, capable of spreading the disease and (2) raising the resistance of the uninfected population to this disease. The first goal can be achieved through the use of effective chemotherapy regimens, the second can be attempted with vaccination programmes involving the attenuated bovine tubercle bacillus (i.e. \textit{M. bovis} BCG).

Anti-tuberculosis chemotherapy aims to achieve the complete eradication of the infectious organism from the patient (thereby curing that individual) and to render the patient non-infectious. Thus, immediately a case of active tuberculosis is diagnosed, through the direct microscopical examination of sputum or other specimens, the infected individual should commence treatment with anti-tuberculosis drugs; particularly as it is now accepted that within c. three weeks of beginning treatment, the infectivity of patients is greatly reduced (British Thoracic Society, 1983). Epidemiologists now consider case-finding and treatment to be the most certain and most rapid means for controlling tuberculosis (Styblo and Meijer, 1978).

In 1980, Mitchison suggested that the tubercle bacilli population of a tuberculous patient could be divided into four groups, based on differences in their metabolic activities. The first group consists of a large number of metabolically active, extracellular bacilli; the second group consisting of a lesser number of moderately
active, intracellular bacilli (i.e. within macrophages); the third group containing both intracellular and extracellular bacilli, showing only occasional bursts of metabolic activity and the fourth group consisting of dormant bacilli, showing no metabolic activity. A consequence of this work was the recognition that therapeutic drug regimens should contain a number of anti-tuberculosis drugs capable of (i) killing those metabolically active bacilli, making up the greater proportion of the tubercle bacilli population and most able to spread the infection through the community and (ii) preventing the emergence of drug-resistant strains of tubercle bacilli (this point had been recognised as long ago as 1946 when, within a few years of the onset of streptomycin-monotherapy, Youmans et al. (1946) detected streptomycin-resistant tubercle bacilli).

In theory the aims of anti-tuberculosis chemotherapy should be achieved in all cases, in practice however, particularly in developing countries, this is not always the case. Among the main reasons for the failure of tuberculosis treatment programmes in these countries are: (1) drug regimens considered ideal for developed countries are often unsuitable for developing countries, particularly on the basis of cost; (2) insufficient supplies of drugs; (3) non-compliance of the patients with the treatment; (4) irregular medical supervision and (5) inadequately trained technical, laboratory and general health staff, involved with case-finding and case-holding. (Joint IUAT/WHO Study Group, 1982). Thus, new regimens need to be developed which will (i) improve the regularity of drug-taking (requiring only intermittent treatment, allowing each dose to be fully supervised) and with (ii)
shorter treatment times, to encourage patients to persevere with their treatment and to keep the cost of treatment down.

The anti-tuberculosis vaccine (BCG) was prepared by Calmette and Guérin from a virulent strain of the bovine tubercle bacillus. These workers subcultured this organism 231 times during a 13 year period (1908-1921), eventually obtaining an attenuated strain (Guérin, 1957). Initially there was a great deal of worry concerning the use of this attenuated strain as a vaccine (worry brought upon by the fear that the organism might revert to virulence) and this worry was fueled by an accident in 1930, when 72 infants died following vaccination with “BCG”, although subsequent investigations revealed that this batch of vaccine had been prepared from a virulent strain of M. tuberculosis. Recently the safety record of the anti-tuberculosis vaccine was reviewed (Lotte et al., 1978) and these authors found that only 31 deaths had been reported (during the period 1948-1974) following the vaccination of c.1.5 billion individuals.

A number of trials have been carried out in different regions of the world to determine the effectiveness of the BCG vaccine in protecting individuals against tuberculosis (and leprosy; section 2.2.4). Table 4 below, summarises the results of seven such trials and shows that in certain regions of the world, BCG vaccination can confer high degrees of protection on susceptible populations. However, as the developing nations have the greatest need for anti-tuberculosis vaccination programmes (these nations have the highest incidences of tuberculosis), the most important trial result and paradoxically the
most worrying was the complete lack of protection obtained in the Madras trial.

Following the failure of the BCG trial in Madras (and to explain the varied protective efficacy shown by the vaccine in different parts of the world) Stanford et al. (1981) have postulated, on the basis of results obtained following skin-test studies in areas where BCG is known to be (i) effective and (ii) ineffective (Paul et al., 1975; Stanford et al., 1976a; Shield et al., 1977), that contact with environmental mycobacteria may predetermine the protective efficacy of BCG. These authors proposed that the cell-mediated immune response to mycobacterial infections is manifest in two forms (with one more protective against mycobacteria than the other): (i) a Koch-type response which is necrotic and non-protective (characterised by its appearance 4-6 weeks after infection; an accumulation of lymphocytes and macrophages at the reaction site (Dienes and Mallory, 1932; Gell and Hinde, 1951; Boughton and Spector, 1963) and a characteristic skin-test time course, which peaks at 40-48 hours and remains high for 96 hours (Rook and Stanford, 1979)) and (ii) a Listeria-type response, able to kill the initial invading bacilli and induce protection to future mycobacterial infections (characterised by its appearance 10-20 days after infection (Rook and Stanford, 1979); an accumulation of lymphocytes and macrophages at the reaction site (Mackaness, 1968) and a characteristic skin-test time-course, which peaks between 18-23 hours and disappears by 48 hours (Rook and Stanford, 1979)).
Moreover, different mycobacteria differ in their ability to induce the Koch- and Listeria-type responses, with fast-growing species only able to induce the Listeria-type response and slow-growing species able to induce both responses, i.e. moderate contact with slow-growing species induces the Listeria-type response but excessive contact induces the Koch-type response (Stanford et al., 1980; Shield, 1983). Thus, Stanford and his colleagues (1981) postulated that (i) where the environmental mycobacteria primed for the Listeria-type response, BCG vaccination would protect those individuals from tuberculosis (and leprosy) and (ii) where the environmental mycobacteria primed for the Koch-type response, BCG vaccination would be ineffective or may even increase the susceptibility to disease.

Using data from the Madras trial, Shield (1983) reported that an extremely high proportion of this study population were infected and sensitised to \textit{M. avium} species (i.e. c.90% were infected and c.98% showed sensitisation to \textit{M. avium}) and in the population showing the greatest incidence of tuberculosis, the greatest percentage of strong and very strong reactions to \textit{M. avium} were observed. Thus, this trial failed because \textit{M. avium} had primed the population for the Koch-type response and BCG vaccination had simply reinforced this non-protective response.
### Table 4: Summary of Results from Seven BCG Vaccination Trials Against Tuberculosis

<table>
<thead>
<tr>
<th>Study Group/Area</th>
<th>Numbers Vaccinated</th>
<th>Follow-up Period (year)</th>
<th>Percentage Protection Against Tuberculosis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Indians</td>
<td>1551</td>
<td>20</td>
<td>80</td>
<td>Aronson et al. (1958)</td>
</tr>
<tr>
<td>Chicago Infants</td>
<td>1716</td>
<td>12 - 23</td>
<td>74</td>
<td>Rosenthal et al. (1961)</td>
</tr>
<tr>
<td>Georgia/Alabama</td>
<td>16913</td>
<td>14</td>
<td>14</td>
<td>Comstock and Palmer (1966)</td>
</tr>
<tr>
<td>Puerto Rico</td>
<td>50634</td>
<td>6 - 7</td>
<td>31</td>
<td>Palmer et al. (1958)</td>
</tr>
<tr>
<td>British Schoolchildren</td>
<td>13598</td>
<td>12 - 15</td>
<td>79</td>
<td>M.R.C. (1963)</td>
</tr>
<tr>
<td>South India (Mandanapalle)</td>
<td>5069</td>
<td>2 - 7</td>
<td>60</td>
<td>Frimodt-Møller et al. (1964)</td>
</tr>
<tr>
<td>South India (Madras)</td>
<td>101106</td>
<td>continuing</td>
<td>0</td>
<td>Tuberculosis Prevention Trial (1980)</td>
</tr>
</tbody>
</table>
2.1.5 Opportunistic Mycobacterial Infections

Unlike *M. leprae* and *M. tuberculosis*, the remainder of the genus *Mycobacterium* live freely in the environment, particularly in watery environments including marshes, streams and even piped water supplies (Collins et al., 1984). However, in common with *M. leprae* and *M. tuberculosis* a number of these environmental mycobacterial species are capable of causing opportunistic disease in man, which can occasionally be severe and even fatal. Although a number of these mycobacterial species had been occasionally isolated from lesions in animals and man earlier, the pathogenic role of environmental mycobacteria was not recognised until *M. ulcerans* and *M. marinum* were demonstrated to be the respective aetiological agents of two distinct diseases: Buruli ulcer (MacCallum et al., 1948) and swimming pool granuloma (Linell and Norden, 1954).

Epidemiologically, diseases caused by opportunist mycobacteria are rarely, if ever, caused by person to person contact. Consequently, measures taken to combat the spread of tuberculosis within the community are ineffective in the control of these diseases, moreover their incidence increases relative to that of tuberculosis, where the latter is in decline. The prevalence of disease due to opportunistic mycobacterial infection is low relative to that of tuberculosis, however Yates et al. (1986) have recently documented that diseases resulting from infection by these organisms, now account for over 5% of all bacteriologically confirmed mycobacterial disease in south-east England. The relative frequency of infections with these opportunist organisms is
determined by their distribution in the environment, consequently in
Britain overall, the predominant species is \textit{M. kansasii}, although in
south-east England \textit{M. xenopi} is the greatest cause of opportunist
mycobacterial disease.

According to Grange (1987), diseases caused by opportunistic
mycobacteria can be divided into two main types: (1) inoculation-type
diseases, resulting in the formation of local lesions and (2) those
diseases caused by inhalation or ingestion of these organisms. Swimming
pool granuloma is the classic example of inoculation disease and results
from infection of previously acquired superficial abrasions by the
opportunist mycobacterium \textit{M. marinum}. Other examples of this type of
disease include post-injection abscesses, typically caused by the
injection of \textit{M. fortuitum} or \textit{M. chelonae} contaminated material (Borghans
and Stanford, 1973) and the inoculation of \textit{M. ulcerans} into the skin and
the formation of a Buruli ulcer (Barker, 1973).

Diseases caused by the inhalation or ingestion of the causative
organisms closely resemble tuberculosis, consequently the organ most
frequently infected with opportunistic mycobacteria is the lung.
Furthermore, in parallel with tuberculosis, the disease may manifest as
localised pulmonary lesions, localised extrapulmonary lesions or be
disseminated. Pulmonary disease is most often caused by the MAIS
organisms (\textit{M. avium-intracellulare-schorfuleceum}) and \textit{M. kansasii} (Grange
and Yates, 1986) and often strikes individuals, susceptible through an
old tuberculous cavity or an immunosuppressive disorder, such as AIDS,
although disease has been known to occur in apparently healthy individuals.

The extrapulmonary opportunist diseases are again similar to tuberculosis and single or multiple localised lesions can occur in bone, urinary tract, central nervous system, lymph nodes and virtually any organ; moreover disseminated disease with multi-organ involvement can also occur. This type of disease usually occurs in association with immunosuppressive disorders, such as congenital defects in cell-mediated immunity and has been observed in AIDS (Iseman et al., 1985). Although this type of disease is at present relatively uncommon, incidences are increasing in frequency and are expected to continue particularly in view of the increasing numbers of AIDS patients.

In contrast to tuberculosis, detection of an opportunist mycobacterium from a clinical specimen, does not indicate disease. Diagnosis is made bacteriologically, requiring the repeated isolation of the organism over several weeks; cultural procedures can unequivocally determine the nature of causative organisms. Typically, the treatment of these opportunist diseases is based on antituberculosis chemotherapy. For the treatment of pulmonary disease due to M.avium-intracellulare, workers have advocated the use of from three to six anti-tuberculosis drugs and for periods of between 2 and 3 years (Dutt and Stead, 1979; Hunter et al., 1981). Other workers have shown that M.kansasii and M.xenopi infections can be effectively treated in 2 years with a triple-drug regimen (Banks et al., 1983; Smith and Citron, 1983). Furthermore, drug regimens have been developed and used with some
success to treat disseminated *M. avium-intracellulare* infections in AIDS patients (Iseman et al., 1985).

In addition to the diseases in which mycobacteria are the obvious causative agents, there are some conditions of unknown aetiology which resemble mycobacterial infections. One of these conditions is Crohn's Disease (Crohn et al., 1932), a granulomatous form of enteritis initially considered to be a clear-cut disease of the terminal ileum but more recently recognised to affect any part of the gastro-intestinal tract from stomach to anus and evidence has been collected showing that the disease may involve the oesophagus, mouth or skin (Mitchell and Rees, 1983).

Mycobacterial involvement in Crohn's Disease (CD) was initially considered, simply on account of the resemblance of this disease to intestinal tuberculosis (Golde, 1968) but failure to isolate mycobacteria from CD tissue led to the demise of this hypothesis. More recently however, a number of groups have reported the isolation of acid-fast bacilli from CD tissue: (i) Burnham et al. (1978) isolated *M. kansasii*; (ii) Chiodini et al. (1984a; b) isolated mycobacteria from three CD patients and biochemical analysis demonstrated that all three species most closely resembled *M. paratuberculosis* [DNA-DNA hybridisation studies confirmed that these three species were indistinguishable from each other and from the type strain of *M. paratuberculosis* (Yoshimura et al., 1987; McFadden et al., 1987a; b)] and (iii) Graham et al. (1987) isolated several different rapid and slow growing mycobacterial species.
from tissue of CD patients. These results have once more raised the possibility of mycobacterial involvement in Crohn's Disease.

2.2 Leprosy

For centuries (prior to 1985 and AIDS) leprosy has been the infectious disease most associated with stigma and fear, a situation related to the fact that leprosy seldom kills but can cause horrific deformities, thus forcing the uninfected population to watch those infected survive but grow steadily worse. *Mycobacterium leprae*, the causative organism of leprosy, was originally identified microscopically by Armauer Hansen in 1873, within the tissues of patients with leprosy and was the first bacterial pathogen of man to be described.

*M. leprae* is an obligatory intracellular parasite and the disease predominantly affects the skin, the peripheral nerves and the mucous membranes of the upper respiratory tract, particularly the nose. Despite its highly infectious nature, only a proportion of individuals infected, actually develop clinical signs of the disease after the usual 3 to 5 years incubation period. The majority of infected individuals seem to develop a subclinical infection which heals spontaneously. Another important aspect of the disease is the variety of clinical symptoms presented by infected individuals, which led Ridley and Jopling (1966) to describe a disease spectrum for leprosy, consisting of polar tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline leprosy (BB), borderline lepromatous leprosy (BL), sub-polar lepromatous leprosy (LLs) and polar lepromatous leprosy (LLp). At one
end of the spectrum, TT leprosy is characterised by localisation of the
disease to one or a few sites in the skin and large peripheral nerves,
furthermore the skin lesions are well-defined and contain very few
\textit{M. leprae} bacilli. At the other end of the spectrum (LL leprosy) the
disease is not localised but spreads rapidly both locally and via the
blood system to other parts of the skin, to nerves, to the mucosa of the
upper respiratory tract and to all the organs of the body. Between
these two extreme forms of the disease, the borderline forms exhibit a
range of intermediate properties, e.g. increases in the numbers of skin
lesions and \textit{M. leprae} bacilli are observed following the spectrum from BT
leprosy through BB leprosy to BL leprosy. These borderline disease
forms are unstable, consequently there is a tendency for infected
individuals displaying borderline symptoms to move in either direction
along the spectrum towards the more stable, polar-forms of the disease;
with treatment, towards the tuberculoid pole, while the untreated
patient tends to move towards the lepromatous pole (Jopling, 1984).

Nerves are affected in all forms of the disease, with the
consequence that destruction of the sensory nerves causes loss of
feeling in the hands and feet which can then be easily damaged.
Invasion of the motor nerves by the leprosy bacillus causes paralysis,
resulting in the loss of movement in the hands and feet. Furthermore,
paralysis of the facial muscles affects expression. Thus it can be seen
how invasion of both the sensory and motor peripheral nerves by \textit{M. leprae}
results in all the characteristic deformities associated with this
disease, i.e. claw hand, drop foot, facies leprasa, anesthesia and
osteoporosis, resulting in the resorption of digits. It is the
secondary injuries sustained by leprosy sufferers, which are essentially the reason for this disease retaining such a high profile in the imagination of the uninfected population and why ostracism remains the fate of many sufferers and their families. Moreover, the social stigma of the disease can inhibit sufferers from seeking medical help before the disease becomes advanced and easily recognisable (Bloom and Godal, 1983).
2.2.1 History of the Disease and its Distribution in the World Today

There is still a great deal of speculation regarding the history of man's relationship with leprosy, concerning the age of the disease and its origins. Gwei-Djen and Needham (1967) and Skinsnes (1980) have each cited literary evidence to suggest that leprosy was established in China during the first millennium B.C. Furthermore, in 1984 Stewart-Tull cited literary evidence to suggest that the disease was present in Egypt c.5000 years B.C. However the earliest physical evidence of leprosy has been supplied by Dzierzykry-Rayalski (1980), when he diagnosed the disease in the skeletons of four adults, from a second century B.C. cemetery in Egypt.

Leprosy, despite its predominance nowadays in tropical and subtropical regions of the world, is not historically a tropical disease. The disease was once rife throughout Northern Europe and as recently as 1860 there were several thousand cases registered in Norway (Bloom and Godal, 1983). Today the disease has almost been totally eradicated in Norway; between 1921 and 1970 only 14 cases were reported (Irgens, 1980). Pockets of endemic leprosy still exist in Europe today and recent WHO figures (Sansarricq, 1981) estimate the number of leprosy cases in Europe to be c.25000. Since 1951, when the disease became notifiable in Britain, there have been 973 registered sufferers in this country, although all have contracted the disease abroad (Browne, 1975). The last recorded case, attributed to endemic transmission in Britain, was in 1798 in the Shetland Isles. In the United States of America, leprosy is endemic in four states: Hawaii, Texas, California and
Louisiana and in the period 1971 to 1981, 1835 cases of the disease were reported, although only 10% of these cases were indigenous (Neill et al., 1985).

According to WHO figures there are an estimated 10-15 million leprosy sufferers in the world today, concentrated mainly in Africa and the Indian subcontinent. Only 5 million of these cases have actually been registered and over one-half of these are concentrated in India (Sansarricq, 1981). In 1982, Sansarricq further reported that leprosy was endemic in 142 countries worldwide and that potentially 2.5 billion people were at risk in contracting the disease.
2.2.2 Immunological Responses to Mycobacterium Leprae Infection

The factor determining a patient's position within the leprosy disease spectrum (described by Ridley and Jopling, 1966), is the extent to which the cell-mediated immunity (CMI) of that individual is expressed. Tuberculoid leprosy (TT) can be characterised immunologically by a high degree of cell-mediated immunity and the presence of numerous lymphocytes within the lesions, whereas lepromatous leprosy (LL), at the other extreme of the spectrum, is characterised by a complete lack of cellular immune responses to the infection. Between these two extreme forms of the disease, the borderline forms exhibit a range of cellular immune responses, reflecting the balance between bacillary multiplication and the degree of cell-mediated immunity expressed by the individual. As with tuberculosis earlier (section 2.1.3), protection against leprosy is cell-mediated, consequently an individual successfully combats an *M. leprae* infection according to those procedures outlined in section 2.1.3 (through which a host overcomes infection by the tubercle bacillus). If the host is unable to mount an effective cell-mediated immune response to combat the *M. leprae* infection, disease ensues.

Two in vitro tests have been applied to leprosy patients to measure their CMI response: the lymphocyte transformation test (LTT), in which T-cells are stimulated to proliferate following contact with *M. leprae* and the leucocyte migration inhibition test (LMIT), which determines the capacity of T-cells to produce migration inhibition factor (MIF) following antigenic stimulation. Lepromatous leprosy
patients show a negative response to the LTT test, whereas tuberculoid leprosy patients are strongly positive and borderline patients show intermediate results. Unfortunately however, the test is not a reliable tool for evaluating individual patients, as negative results have also been recorded with borderline tuberculoid patients and strong responses demonstrated with some borderline lepromatous patients (Ridley, 1976). The LMIT test has also been shown to produce negative responses from lepromatous patients and positive responses with tuberculoid patients (Jopling, 1984).

The lepromin skin-test is also used for demonstrating impairment of the CMI response of leprosy sufferers and a number of different reagents have been described and used: Mitsuda lepromin, consisting of heat-killed *M.leprae* bacilli, extracted from human tissue; Dharmendra lepromin, consisting of a more purified suspension of heat-killed *M.leprae* bacilli, extracted from human tissue and lepromin A, an autoclaved suspension of *M.leprae* infected tissue derived from armadillos. A positive lepromin skin-test is biphasic, with an early and late reaction. The early, Fernandez reaction, appears within 24 to 48 hours of injection and is most readily seen with the Dharmendra lepromin; the late, Mitsuda reaction, occurs between 2 and 4 weeks after injection and is most noticeable when either of the two cruder skin-test reagents are used (Mitsuda lepromin and lepromin A). Unfortunately, skin-testing cannot be used to diagnose leprosy, as healthy individuals often produce positive skin-test responses, even in areas of the world where leprosy is non-endemic. The test however is of value for classifying diagnosed cases: tuberculoid leprosy patients

- 40 -
(TT) produce positive responses; borderline patients produce intermediate responses and lepromatous leprosy patients (LL) are unresponsive. Consequently, a positive Mitsuda reaction is indicative of an individual's ability to mount an effective cell-mediated response to combat infection with M. leprae.
For centuries leprosy control consisted of separating leprosy sufferers from the general population and evidence from Dzierzykry-Rogalski (1980) suggested that segregation was practised as early as the second century B.C. However the effectiveness of isolation in reducing leprosy incidence rates is debatable whereas its role in accentuating fear and prejudice within the uninfected community is unquestionable.

Historically, the study of leprosy has suffered from the inability to obtain sufficient quantities of \textit{M. leprae} bacilli to work with, a situation exacerbated by the continued failure to culture \textit{M. leprae} bacilli on artificial media. Recently however, advances have been made in the development of animal models, for the cultivation of \textit{M. leprae} bacilli. In 1960, Shepard used the mouse footpad as the site for \textit{M. leprae} inoculation and demonstrated for the first time, the successful transmission of human leprosy to a laboratory animal. Rees (1966) expanded this technique and established enhanced growth of \textit{M. leprae} bacilli in immune-deprived mice. However, the greatest breakthrough in the cultivation of \textit{M. leprae} bacilli came when Kirchheimer and Storrs (1971) demonstrated that the nine-banded armadillo (\textit{Dasypus novemcinctus} Linn) was naturally highly susceptible to \textit{M. leprae} infection and produced substantial numbers of bacilli following c.15 months of infection. More recently, Wolf et al. (1985) have reported the transmission of leprosy to three monkey species (sooty mangabeys, rhesus monkeys and African green monkeys), thus providing an animal model that resembles the human disease more closely. To date
however these experimental animal infections provide the only readily available source of *M. leprae* bacilli but do increase the likelihood of a successful outcome to the search for anti-leprosy drugs and a specific anti-leprosy vaccine.

**Chemotherapy**

Chemotherapy of leprosy patients began in the early 1940's with the use of Promin (Faget et al., 1943), however by the late 1940's dapsone had replaced this sulphone derivative. Dapsone (4,4' diaminophenyl sulphone; Figure 1.), with its bacteriostatic action against *M. leprae*, inhibiting the enzyme dihydropteroate synthase (DHPS); its low cost, e.g. $5.00 per patient per year (Bloom and Godal, 1983); its low toxicity and lack of side-effects was hailed as an anti-leprosy "wonderdrug" and eradication of leprosy was widely anticipated.

**Figure 1.** : *Structure of Dapsone* (Winder, 1983)
For 40 years dapsone-monotherapy has remained the central focus of leprosy control programmes throughout the world, however the anticipated eradication of leprosy has never occurred, instead the WHO has labelled dapsone-monotherapy a failure. According to the WHO, failure of the leprosy control programme was largely due to poor monitoring of patients undergoing treatment, resulting in patients not complying with the rigorous demands of treatment (tuberculoid patients were expected to undergo dapsone-monotherapy for 2 to 3 years and lepromatous patients were expected to continue treatment for life), and their subsequent relapse within a year or two. Furthermore even patients who had diligently followed their treatment protocol were prone to relapse, Waters et al. (1974) found small numbers of viable, dapsone-sensitive bacilli in patients after 10 years of dapsone-monotherapy. A further problem created by intermittent dapsone-monotherapy was the establishment of highly resistant populations of *M.leprae* bacilli (secondary dapsone resistance). This phenomenon was first reported by Pettit and Rees (1964) and at that time appeared to be only a minor problem, with an occurrence of approximately 2 cases per 1000. However in 1973, Meade et al. reported the occurrence of secondary dapsone resistance to be as high as 25 cases per 1000. Since 1977, secondary dapsone resistance has become such a major problem that wherever in the world it has been sought, cases have been detected. Primary dapsone resistance, on the other hand, is diagnosed when persons, previously untreated with dapsone, are found to be unaffected by dapsone monotherapy. In 1977 (Pearson et al., 1977) the existence of primary dapsone resistance was documented for the first time.
When the WHO Fifth Expert Committee on Leprosy met in 1976, secondary dapsone resistance was considered such a major problem, recommendations were made that all active cases of lepromatous leprosy be treated with at least two anti-leprosy drugs. However, at that time there was too little experience with multi-drug therapy for definitive regimens to be recommended, consequently different workers used a variety of drug regimens to combat the disease. In 1981 the WHO convened a Study Group, to review the multitude of information obtained concerning the use of multidrug regimens for all types of leprosy patients, whether newly diagnosed, apparently successfully treated or relapsed.

Following the 1981 meeting, the WHO Study Group recommended that triple-drug therapy should be employed against leprosy, with dapsone included in the regimen on account of its low cost, low toxicity and lack of side-effects. The second anti-leprosy drug recommended for inclusion in the new treatment programme was rifampicin (Figure 2). This antibiotic binds to the enzyme RNA polymerase, resulting in the formation of a stable drug-enzyme complex which inhibits RNA synthesis, subsequently killing the infecting bacilli (Winder, 1983). The potent bactericidal effect of this drug against M. leprae was demonstrated by Rees et al. (1970); a single dose of rifampicin accomplished as much killing of M. leprae bacilli in a few weeks as dapsone achieved in a few months. Furthermore these authors found rifampicin to be effective against dapsone-resistant M. leprae also.
A third drug was considered necessary to prevent the emergence of *M. leprae* strains resistant to both dapsone and rifampicin, particularly following the detection of a rifampicin-resistant strain of *M. leprae* by Jacobson and Hastings (1976). The recommended third drug was clofazimine (Figure 3), a highly effective anti-leprosy drug, first used by Browne and Hogerzeil in 1962. There is however, an unfortunate side-effect associated with this drug, abnormal skin pigmentation, which is particularly noticeable in light-skinned patients. Consequently
where the use of clofazimine proves unacceptable the Study Group suggested the use of the anti-tuberculosis drug ethionamide.

Figure 3. : Structure of Clofazimine (Winder, 1983)

This new triple-drug regimen was recommended for lepromatous leprosy patients and furthermore therapy was recommended to be administered for at least 2 years, continuing until skin-smears were negative. The Study Group also recommended that tuberculoid leprosy patients be treated with two drugs, dapsone and rifampicin, and that treatment of these individuals be continued for 6 months. This particularly short time-scale was recommended following the success
achieved by Warndorff et al. (1982) in treating tuberculoid patients with rifampicin.

It is hoped, that through these two new, multidrug regimens, with their shorter treatment times: (i) more leprosy sufferers will be encouraged to register for treatment and (ii) a rapid turnover of successfully treated patients will ensue, which will keep morale high amongst those patients still undergoing treatment and furthermore encourage their continued compliance with the treatment programme. Moreover, it is anticipated that reductions will finally be seen in the number of registered cases, particularly registered tuberculoid leprosy cases, which amount to almost 80% of the total number of registered cases (Waters, 1983).
2.2.4 Vaccination Against Leprosy

From the general experience of the control and eradication of other infectious diseases, it would appear unlikely that chemotherapy alone, even in the form of the new multidrug regimens recommended by the WHO, will result in the eradication of leprosy. Experience suggests that two concerted approaches are required: (1) chemotherapy, to treat those individuals with the disease and to correspondingly reduce their ability to spread the disease and (2) an effective anti-leprosy vaccine to protect the uninfected population.

Prior to 1971 and the cultivation of \textit{M. leprae} bacilli in the nine-banded armadillo (Kirchheimer and Storrs, 1971), the possibility of producing a specific anti-leprosy vaccine had been considered extremely unlikely. Nevertheless vaccination has been attempted against leprosy. As early as 1939, Fernandez vaccinated healthy, lepromin-negative children with BCG (cited by Lwin et al., 1985), noted lepromin conversion in over 90% of these individuals and subsequently concluded that BCG may confer some protection against leprosy. A number of other small-scale studies, using BCG as a vaccine against leprosy, followed on from the initial work by Fernandez, i.e. De Souza Campos, 1953; Fernandez, 1955; Convit, 1956; Chatterjee et al., 1958; and Yanagisawa, 1960 (all cited in Lwin et al., 1985) and protection against leprosy was found to range from 26 to 96%. However, these early trials with BCG were neither on a large enough scale nor well enough controlled.
to withstand critical analysis and consequently doubts remained, concerning the true value of BCG vaccination against leprosy.

However, since 1960, five major trials have been undertaken to evaluate the protective effect of BCG against leprosy, i.e. in Uganda; Burma; Papua New Guinea; India and Malawi. (1) The Ugandan trial began in 1960 and involved over 19000 children, aged 0-14 years, who were all relatives or contacts of known leprosy sufferers. (Brown and Stone, 1966). Following vaccination with BCG the subjects were assessed for 8 years and the protective effect of BCG against leprosy was found to be 80% (Stanley et al., 1981). (2) The Burmese trial involved c.26000 children aged 0-14 years (Bechelli et al., 1970) and after 14 years of follow-up, BCG was shown to have a protective effect of only 20% (Lwin et al., 1985). (3) The Papua New Guinea trial involved almost 5000 subjects, of all ages (Russell et al., 1964) and BCG showed a protective effect of 46% during a 10 year follow-up period (cited by Lwin et al., 1976). (4) The Indian trial involved 180000 subjects and after 10 years the protective effect of BCG was 23% (Tripathy, 1984). (5) The trial in Malawi involved 112000 individuals and BCG showed a protective effect of at least 50% (Fine et al., 1986). With BCG protection against leprosy found to vary from 20-80% in these five trials, these trials confirmed the need for a specific anti-leprosy vaccine.

Since 1971 and the use of the nine-banded armadillo for the large-scale cultivation of M. leprae bacilli, the development of a specific anti-leprosy vaccine has become possible. In 1974 IMMLEP, the
Scientific Working Group on the Immunology of Leprosy (part of the UNDP/World Bank/W.H.O. Special Programme for Research and Training in Tropical Disease) made their priority the establishment of large colonies of *M. leprae*-infected, nine-banded armadillos. By 1982, the IMMLEP Programme had achieved the infection of over 300 armadillos in centres in the USA and Britain; developed a protocol for the extraction and purification of large quantities of *M. leprae* bacilli from armadillo tissue and produced a standard preparation of *M. leprae* bacilli, suitable for vaccine trials in man. (The IMMLEP candidate anti-leprosy vaccine consists of heat-killed, gamma-irradiated, whole *M. leprae* bacilli.)

There are two rationales for vaccination against leprosy, one is immunoprophylaxis, designed to protect a population at risk against developing clinical leprosy and the second is immunotherapy, designed to convert lepromatous patients to a state of cell-mediated immune responsiveness (Bloom and Mehra, 1984). A killed *M. leprae* vaccine would be exclusively suited for immunoprophylaxis (lepromatous leprosy patients are immunologically unresponsive to *M. leprae* antigens) and the premise is that a naive population, vaccinated with killed *M. leprae*, would be primed to positive immune reactivity to specific *M. leprae* antigens. Thus, if at a future date, these individuals become infected with *M. leprae*, the infecting bacilli will serve to boost the already pre-existing level of cell-mediated immunity and these persons would develop either subclinical leprosy and eliminate the organism or develop the polar tuberculoid (TT) type of self-healing disease.
The basis for a vaccine of killed *M. leprae* plus live BCG (to be used in immunotherapy) derives from observations by Convit et al. (1974), that *M. leprae*, when injected into the skin of lepromatous patients together with BCG, caused degradation and clearance of *M. leprae* bacilli, which was not seen when *M. leprae* was inoculated alone. These results provided the experimental basis for the use of this mixture of mycobacteria in studies of immunotherapy (and immunoprophylaxis) in leprosy by Convit and his colleagues (Convit et al., 1979; 1982). These authors subsequently demonstrated that their vaccine (heat killed *M. leprae* plus BCG) produced a therapeutic effect on intermediate, borderline and lepromatous leprosy patients, including skin-test conversion, degradation and clearance of *M. leprae* bacilli from skin lesions and clinical improvement. These results highlighted the potential a combined *M. leprae*/BCG vaccine has in the treatment of highly susceptible individuals or those already infected.

Recently, two large-scale trials of the IMMLEP vaccine have begun, in two different regions of the world (past experience with the BCG vaccination trials against leprosy showed that it was impossible to extrapolate results from one region of the world to another). In 1984, over 60000 contacts of 2000 leprosy patients began registering for entry into a vaccine trial in Venezuela. All household contacts aged six years or more were entered into the trial, as were nonhousehold contacts with negative skin-tests and it was anticipated that 30000 subjects would participate, with half the subjects receiving BCG + the IMMLEP vaccine (6 x 10⁶ killed, *M. leprae* bacilli) and the other half receiving BCG alone. Examination of all the subjects (for the development of
leprosy) is to be carried out at one-yearly intervals and chemotherapy offered to those diagnosed positive (Zuniga and Convit, 1985). The second large-scale IMMELP vaccine-trial was announced by LEPPRA (the British Leprosy Relief Association) in 1985 and will involve 120,000 subjects from the Karonga district of Malawi (Williams, 1985). Once again, this trial will compare the incidence of leprosy in persons vaccinated with BCG + the IMMELP vaccine (5 x 10^7 killed, M.leprae bacilli) versus those vaccinated with BCG alone. In both trials, follow-up is expected to continue for a number of years.

In India, two cultivable mycobacterial strains have also been reported to be effective at inducing cell-mediated immunity in lepromatous leprosy patients. Deo et al (1981), using a killed, cultivable mycobacterium (the ICRC bacillus; a member of the M. avium-intracellulare-sorofulaceum group) reported over 90% skin-test conversion in (i) lepromin-negative, healthy individuals and in (ii) borderline (BB) and borderline lepromatous (BL) patients. Similarly, Chaudhuri et al. (1983) achieved significant skin-test conversion rates in normal individuals and lepromatous leprosy patients with Mycobacterium V (another member of the M. avium-intracellulare-sorofulaceum group).

With armadillos the only major source of M.leprae bacilli to date, one appealing aspect of immunotherapy involving the ICRC bacillus or Mycobacterium V is the potential to produce very large quantities of these two cultivable organisms cheaply. Moreover, Emmrich and Kaufmann (1986) recently prepared human T-cell clones from tuberculoid leprosy
patients and compared the ability of the anti-leprosy vaccine strains of *M. leprae*, BCG and the ICRC bacillus to stimulate these T-cells. These authors subsequently reported that superior stimulation of the T-cell clones was obtained with the ICRC bacillus and concluded that this result favoured the use of this organism as an anti-leprosy vaccine. Presently, a large-scale trial of the ICRC vaccine is underway in India.
Section 3  Molecular Biology and Mycobacteria

In 1983 a joint meeting of IMMLEP and IMMTUB Scientists was convened to assess the role molecular biology could play in combatting both tuberculosis and leprosy. The subsequent outcome of this meeting was that the following goals were set for this new technology: the cloning of *M. tuberculosis* and *M. leprae* genes into more convenient and easier handled bacterial hosts, in order to overcome the extremely slow rates of growth of these two organisms and the major hindrance to their study (particularly important for *M. leprae* which has yet to be cultured in vitro); the expression of mycobacterial gene products and the identification of molecules to be used as skin-test reagents for assessing cell-mediated immunity in susceptible individuals; the preparation of purified antigens for the development of antigenic vaccines and also, the cloning and characterisation of unique mycobacterial enzymes, against which drugs could be targeted, allowing the subsequent development of new chemotherapy regimens.

Clark-Curtiss et al. (1985) described the construction of an *M. leprae* genomic DNA Library using the *E. coli* cosmid vector pHC79 and their subsequent screening of these recombinant clones for the complementation of a number of genetic defects in the host *E. coli* strains. However their inability to detect any complementation had already raised doubts as to the ability of the *E. coli* transcription and translation apparatus to recognise mycobacterial promoter signals. These authors were only able to detect expression of mycobacterial DNA, when cloned into a plasmid expression vector containing a strong
promoter (the aspartate semialdehyde dehydrogenase (asd) gene promoter from *S. mutans*, which has a strong affinity for *E. coli* RNA polymerase).

These initial doubts were dispelled when a number of authors demonstrated that mycobacterial promoters were functional in *E. coli*: Thole et al. (1985) described the detection of a recombinant EMBL3 clone expressing a 64KD *M. bovis* BCG antigenic protein, from its own mycobacterial promoter; Labidi et al. (1985) demonstrated that a cloned *M. fortuitum* plasmid was capable of expressing a 64KD protein in *E. coli*, from its own promoter; Jacobs et al. (1986) reported the detection of a recombinant *M. leprae* clone expressing a 46KD *M. leprae* protein, able to complement a mutation in the citrate synthase gene of *E. coli* and Kieser et al. (1986) reported the cloning of *M. bovis* BCG DNA fragments into an *E. coli* promoter-probe plasmid (containing a promoterless chloramphenicol-resistance gene) and their subsequent detection of transformants with resistance to chloramphenicol (a result of cloned promoter activity) moreover, their results showed that the majority of *M. bovis* BCG promoters are only weakly active in *E. coli*. More details of this study are described in this thesis.

Kieser et al. (1986) also described the cloning of *M. bovis* BCG DNA fragments into an *S. lividans* promoter-probe vector and demonstrated that *S. lividans* efficiently utilises a high proportion of *M. bovis* BCG promoters. Furthermore these authors described the cloning of *M. bovis* BCG DNA fragments into a translational fusion vector and reported that *S. lividans* uses *M. bovis* BCG translational signals almost as efficiently as its own signals, concluding that *S. lividans* may be a suitable host.
for achieving the expression of \textit{M. tuberculosis} and \textit{M. leprae} genes from their own signals.

The poor recognition of mycobacterial transcription and translation signals by \textit{E. coli} was circumvented by Young et al. (1985a; b) when they cloned \textit{M. tuberculosis} and \textit{M. leprae} genes into the phage lambda vector gtII. This cloning system allows foreign DNA fragments to be placed under the control of the \textit{E. coli} lacZ gene (coding for beta-galactosidase), which ensures that the foreign DNA will be efficiently transcribed and translated in \textit{E. coli}. Furthermore, foreign DNA cloned into this vector has the potential to be expressed as a fusion protein with the expressed beta-galactosidase protein and these fusion proteins are often highly stable and more resistant to proteolytic degradation than is the foreign protein alone.

The gtII/\textit{M. tuberculosis} libraries prepared by Young et al. (1985a) and Husson and Young (1987) were screened with a number of mouse anti-\textit{M. tuberculosis} monoclonal antibodies and clones expressing \textit{M. tuberculosis} proteins of 12KD, 14KD, 19KD, 65KD and 71KD were detected. Young et al. (1987a) further described the screening of a gtII/\textit{M. tuberculosis} library with rabbit anti-\textit{M. tuberculosis} hyperimmune serum and reported the detection of 29 clones, 22 of which expressed three antigens (14KD, 65KD and 71KD) that had previously been identified with mouse monoclonal antibodies (as described above) and concluded that these three antigens are immunodominant as regards the antibody response to mycobacterial proteins. The screening of the gtII/\textit{M. leprae} library (Young et al., 1985b), with a series of thirteen mouse anti-\textit{M. leprae
monoclonal antibodies, revealed clones expressing *M. leprae* proteins of 12KD (the nature of this clone will be described in the Discussion), 18KD, 28KD, 36KD and 65KD, considered to be the five most immunogenic *M. leprae* proteins, by these authors.

The DNA inserts of clones expressing the 65KD *M. leprae* antigen; the 65KD *M. tuberculosis* antigen and the 65KD *M. bovis* BCG antigen have each been sequenced. Mehra et al. (1986) isolated, then sequenced the 3.6Kb DNA insert of a gtII/*M. leprae* clone (gtII/*M. leprae* clone Y3178) containing the entire coding sequence gene of the *M. leprae* 65KD antigen. Within the 3.6Kb of sequenced DNA, two open reading frames were detected and the corresponding amino acid sequences were subsequently deduced for each. One open reading frame encoded for a polypeptide of 588 amino acids (c.62KD) and the second encoded for a polypeptide of 541 amino acids (c.57KD).

Shinnick (1987) screened a gtII/*M. tuberculosis* library with three mouse monoclonal antibodies, specific for the 65KD *M. tuberculosis* antigen and detected 38 clones expressing this antigen. Insert DNA was isolated from 20 of these recombinant clones and a restriction enzyme cleavage site map was initially deduced for the 65KD gene. Several of these insert fragments (comprising between them, the entire gene) were then subcloned into the plasmid pUC19 and were subsequently sequenced. The c.4.4Kb of DNA sequenced, contained eight open reading frames, two of which encoded for polypeptides of 540 amino acids (c.55KD) and 517 amino acids (c.51KD). The remaining six open reading frames, encoded for polypeptides of 142-187 amino acids. Subsequent work by the author
revealed that the 540 amino acid polypeptide reacted with monoclonal antibodies specific for the 65KD *M. tuberculosis* antigen and concluded that this 540 amino acid sequence encoded for the 65KD *M. tuberculosis* antigen.

From a previously selected clone (Thole et al., 1986), expressing the 64KD *M. bovis* BCG antigen, Thole et al. (1987) extracted a 2.4Kb insert fragment comprising the entire 64KD gene. This insert was then subcloned into the vectors pEMBL8 and pEMBL9, to enable both strands of the insert DNA to be sequenced. A number of deletion mutants were subsequently derived from the two pEMBL subclones, using restriction enzymes; DNaseI and Bal31, and these were then sequenced by the Sanger Technique. Within the 2.4Kb nucleotide sequence subsequently obtained, only one large open reading frame was found and this 1617bp open reading frame encoded for a polypeptide of 539 amino acids with a calculated molecular weight of c.57KD.

Comparison of the amino acid sequences deduced for the 64-65KD *M. bovis* BCG, *M. tuberculosis* and *M. leprae* antigens has revealed that the *M. bovis* BCG and *M. tuberculosis* sequences are 100% homologous and furthermore they show extensive homology with the 541 amino acid sequence deduced for the *M. leprae* 65KD antigen (when translation of this antigen occurs from the second open reading frame). However three particular areas were detected within the amino acid sequence of the *M. leprae* antigen, where homology with the *M. bovis* BCG/*M. tuberculosis* amino acid sequence is poor and these areas correspond to three of the six monoclonal antibody recognition sites described for the *M. leprae*
65KD antigen by Mehra et al. (1986). Using eleven monoclonal antibodies that recognise a 65KD mycobacterial antigen, Husson and Young (1987) subsequently showed that seven of these antibodies reacted with the 65KD antigen from both \textit{M. tuberculosis} and \textit{M. leprae}, one antibody reacted with the \textit{M. tuberculosis} antigen only and two antibodies reacted with the \textit{M. leprae} antigen only. Thus with respect to each other, these two antigens have epitopes that are species specific.

Further analysis of the 65KD \textit{M. leprae} antigen was undertaken by Buchanan et al. (1987) with the aid of 23 monoclonal antibodies, all of which recognised this antigen. These workers subsequently identified fourteen different epitopes within this antigenic molecule. These fourteen distinct monoclonal antibodies were additionally used to compare the species distribution of each of the fourteen different epitopes among 23 species of mycobacteria and the results confirmed the 65 KD antigen to be a common mycobacterial antigen. Further details of this point are included in the Discussion. One of the monoclonal antibodies recognised an epitope found only on the 65KD \textit{M. leprae} antigen and these authors, amongst others (Mehra et al., 1986; Shinnick, 1987) have suggested the possibility of using synthesised peptides, corresponding to species-specific portions of the 65KD antigen as tools in immunodiagnostic tests for the early screening of susceptible populations to diagnose leprosy.

With immunity against leprosy and tuberculosis known to be cell-mediated (Hahn and Kaufmann, 1981) a number of workers have attempted to detect antigen-reactive T-cell clones to aid the identification of
M. leprae and M. tuberculosis antigens that are involved in protective immunity. Using the 64KD M. bovis BCG antigen, prepared by Thole et al. (1985), Emmrich et al. (1986) observed that this antigen was capable of inducing proliferation of helper T-cell clones prepared from a tuberculoid leprosy patient and similarly Oftung et al. (1987) reported the preparation of helper T-cell clones from a tuberculosis patient capable of recognising the 65KD M. tuberculosis antigen. Mustafa et al. (1986) have also described the production of M. leprae-specific T-cell clones from M. leprae-vaccinated volunteers and the subsequent stimulation of half of these clones with an antigenic determinant of an 18K M. leprae antigen, previously described by Young et al (1985b). Ottenhoff et al. (1986a) cloned M. leprae-reactive T-cells from a tuberculoid leprosy patient and then reported the detection of a number of helper T-cell clones which were stimulated to proliferate by a 36KD M. leprae antigen. Further work however, by Ottenhoff et al. (1986b) revealed that this 36KD M. leprae antigen could also stimulate suppressor T-cell clones and that these clones were capable of suppressing the helper T-cell responses to the 36KD M. leprae antigen, which had been observed previously by these workers (Ottenhoff et al., 1986a).

Consideration of the results described above, has led a number of workers to suggest a role (as candidate vaccines) for those antigenic epitopes capable of stimulating helper T-cells but not able to stimulate suppressor T-cells.
Section 4  Aims of the Project

The continued inability to culture *M. leprae* in vitro has greatly hindered (i) studies on the biochemistry, physiology and genetics of this organism and (ii) efforts to produce an anti-leprosy vaccine. Presently, experimental infections of nine-banded armadillos (*Dasypus novemcinctus* Linn) provide the major source of *M. leprae* bacilli for research however, this system is extremely expensive, time-consuming and not very efficient.

Gene cloning provides a way of producing foreign proteins in more convenient organisms and moreover provides a means to examine the antigenic proteins of pathogens, allowing the identification and selection of antigens which could be used in the development of vaccines. Furthermore, gene cloning offers the potential to produce very large quantities of important antigens cheaply.

Thus, using gene cloning techniques, the aims of the project were: to develop techniques for the extraction and cloning of mycobacterial DNA; to confirm the suitability of *E. coli* as a host for the cloning and expression of mycobacterial genes and to identify mycobacterial antigens with the potential to function as tools in the diagnosis of leprosy.
Section 5. MATERIALS

5.1 Strains

All the strains used in this work have been listed in Tables 5A and 5B.

Table 5A: Escherichia coli Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB101</td>
<td>F-, proA2, recA, hsdR-, hsdM-</td>
<td>Boyer &amp; Roulland-Dussoix, 1969</td>
</tr>
<tr>
<td>JM101</td>
<td>supE, (lac-proAB), [F',traD36, proAB,lacI^Z M15]</td>
<td>Yanisch-Perron et al, 1985</td>
</tr>
<tr>
<td>NM539</td>
<td>supF, hsdR-, (P2co3)</td>
<td>Frischauf et al, 1983</td>
</tr>
<tr>
<td>Y1088</td>
<td>supE, supF, hsdR-, hsdM-, lacU169, (proC::Tn5) (pmc9)</td>
<td>Young &amp; Davis, 1983b</td>
</tr>
<tr>
<td>Y1089</td>
<td>lacU169, lon, hflA150, [chr::Tn10] (pmc9)</td>
<td>Young &amp; Davis, 1983b</td>
</tr>
<tr>
<td>Y1090</td>
<td>lacU169, lon, supF, [trpC22::Tn10] (pmc9)</td>
<td>Young &amp; Davis, 1983b</td>
</tr>
</tbody>
</table>

(* only the relevant characteristics of each strain have been listed)
### Table 5B: Mycobacterial Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em> BCG (Glaxo)</td>
<td>J.A. Morris; Central Veterinary Laboratory, Weybridge.</td>
</tr>
<tr>
<td><em>M. lepra</em></td>
<td>M.J. Colston; National Institute for Medical Research, Mill Hill.</td>
</tr>
<tr>
<td><em>M. &quot;lufu&quot;</em></td>
<td>M.J. Colston; National Institute for Medical Research, Mill Hill.</td>
</tr>
<tr>
<td><em>K. phlei</em></td>
<td>Departmental Culture Collection</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>Departmental Culture Collection</td>
</tr>
<tr>
<td><em>M. vaccae</em></td>
<td>M.J. Colston; National Institute for Medical Research, Mill Hill.</td>
</tr>
</tbody>
</table>

#### 5.2 Antibiotics

Antibiotics were purchased from Sigma.

Stock solutions of ampicillin (5mg/ml) and tetracycline (10mg/ml) were prepared with distilled water. Chloramphenicol (75mg/ml) was prepared with ethanol.

#### 5.3 Media

**L-Broth**

L-Broth was prepared in accordance with the following recipe:
15g Tryptone  
5g Yeast extract  
5g Sodium chloride

The volume was made up to one litre with distilled water and the pH was adjusted to 7 with sodium hydroxide. Aliquots were dispensed then autoclaved. Supplements (e.g. antibiotics) were added, when appropriate, immediately prior to use.

L-Agar

100ml volumes of L-Broth were prepared (as described above) and dispensed into 250ml Duran bottles, together with 1.5 grams of Agar Technical No. 3. and the mixtures were then autoclaved. Antibiotics were added, as required, immediately prior to use.

T-top agar

15g Tryptone (Oxoid)  
5g Sodium chloride  
8g Agar Technical No. 3 (Oxoid)  
2.46g Magnesium sulphate

The volume was made up to 1 litre with distilled water, then stirred on a magnetic hot plate to melt the agar and 3ml volumes were aliquoted into small glass bottles (Bijoux) and autoclaved.
Sauton medium

0.5g $K_2HP0_4$
2.0g Citric acid
0.5g $MgSO_4.7H_2O$
0.05g Ammonium ferric citrate
20.0g Glycerol
0.2g Tween 80
4.0g Asparagine

The volume was made up to 1 litre with distilled water and the pH was adjusted to 7 with sodium hydroxide. Volumes of 250ml were dispensed into 500ml flasks, then autoclaved.
5.4 Buffers

1M Tris-HCl Buffers

A 2M stock of Tris-base was prepared with distilled water (242.22g per litre). Aliquots were adjusted to the appropriate pH with concentrated hydrochloric acid and distilled water was added to produce a buffer with a final Tris concentration of 1M.

1X T.E. Buffer

- 10mM Tris-HCl pH 8.0
- 1mM EDTA

1X T.E.S. Buffer

- 10mM Tris-HCl pH 8.0
- 100mM NaCl
- 1mM EDTA

SM Buffer

- 200mM NaCl
- 1mM MgSO₄·7H₂O
- 50mM Tris-HCl pH 7.5
- 0.01% Gelatin
The volume was made up to 1 litre with distilled water and then autoclaved.

2 x Freezing Buffer (for storing E.coli cells)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>12.60g</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.90g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.18g</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>1.80g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>3.60g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>88.00g</td>
</tr>
</tbody>
</table>

The volume was made up to 1 litre with distilled water and 100ml volumes were autoclaved.

E.coli cells were suitable for storage at -70°C following the addition of an equal volume of the 2 x Freezing buffer to the E.coli cell suspension.

Restriction Endonuclease Digestion Buffers (Maniatis et al., 1982)

10X Low-salt buffer (10X LQV)

- 10mM Tris-HCl pH 7.5
- 10mM MgCl$_2$
- 1mM Dithiothreitol (DTT)
10X Medium-salt buffer (10X Med)

10mM Tris-HCl pH 7.5
10mM MgCl₂
1mM DTT
50mM NaCl

10X High-salt buffer (10X Hi)

50mM Tris-HCl pH 7.5
10mM MgCl₂
1mM DTT
100mM NaCl

10X Sma1 Digestion buffer (10X Sma)

10mM Tris-HCl pH 8.0
10mM MgCl₂
1mM DTT
20mM KCl

10X Ligation buffer (Maniatis et al., 1982)

660mM Tris-HCl pH 7.6
50mM MgCl₂
50mM DTT

Small volumes were dispensed and stored at -20°C.
5.5 Chemicals

BDH;

Bromophenol blue, Disodium hydrogen phosphate,
Acrylamide (Electran), Ammonium ferric citrate, Ammonium
persulphate (Analar), Ammonium sulphate (Analar), Boric acid (Analar),
Calcium chloride (Analar), Dimethyl sulphoxide (Analar), D-Glucose
(Analar), Glycine (Analar), Hydrogen peroxide (30%), Magnesium sulphate
(Analar), Maltose (Analar), PAGE-Blue 83 (Electran), Phenol (Analar),
Polyethylene glycol 6000 (PEG), Polyoxyethylene sorbitan mono-oleate
(Tween 80), Potassium dihydrogen orthophosphate, Sodium acetate, Sucrose
(Analar), TEMED (N,N,N',N'-Tetramethylethylene-diamine), Tri-sodium
citrate (Analar)

BCL (Boehringer Mannheim);

Caesium chloride (Analar), EDTA (Ethylene diamine tetra-acetic
acid, disodium salt) (Analar)

BRL (Bethesda Research Laboratories Inc.);

Agarose (Ultra-pure and Electrophoresis grade)

Bio-Rad;

Protein Assay

Fisons;

Glycerol
Hopkin and Williams Ltd.

Amido black 10B, Magnesium chloride (Analar), Potassium chloride (Analar), Sodium hydroxide (Analar),

M and B Ltd.

Chloroform (Pronalys AR), Dipotassium hydrogen phosphate, Ethanol (Pronalys AR), Glacial Acetic Acid (Pronalys AR), Hydrochloric Acid (Pronalys AR), Isopropanol (Pronalys AR), Methanol (Pronalys AR), Sodium chloride (Pronalys AR)

Oxoid

Agar technical (Number 3), Gelatin, Tryptone, Yeast extract

Sigma

Acetyl Coenzyme A (lithium salt), Ampicillin (sodium salt), Arginine (freebase), L-Asparagine (monohydrate), Brij 58 (Polyoxyethylene 20 cetyl ether), BSA (Bovine serum albumin, fraction V), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), Chloramphenicol, 4-Chloro-1-naphthol, DL-Dithiothreitol (DTT), Deoxyribonuclease I, DTNB (5,5'-dithiobis -[2 nitrobenzoic acid], Ethidium bromide, IPTG (Isopropyl-Beta-D-thio-galactopyranoside), Lysosyme (grade 1), 2-Mercaptoethanol, N,N'-Methylene-bis-Acrylamide, MOPS (3-[N-Morpholino]propane sulphonic acid), NBT (Nitroblue terazolium), PEG (Polyethylene
glycol), Proteinase K, Ribonuclease A (type ), SDS (Sodium dodecyl
sulphate ), Spermine (Tetrahydrochloride), tetracycline (hydrochloride),
Trizma base (reagent grade), Tween 20 (Polyoxyethylene sorbitan
monolaurate), X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranos-
side)

Koch Light Laboratories

Rubidium chloride

Kodak

Sodium tri-isopropyl naphthalene sulphonate.
5.6 Molecular Biology and Immunochemical Reagents

[alpha $^{32}$S]-Deoxyadenosine Triphosphate, Triethyl-ammonium salt; phage lambda vector EMBL4 and streptavidin-biotinylated peroxidase complex were purchased from Amersham International plc.

Restriction enzymes were purchased from BCL or BRL.

Calf Intestinal Alkaline Phosphatase (CIAP) and T4-DNA ligase were purchased from BCL.

DNA polymerase I (Klenow fragment) and pBR322 DNA were purchased from BRL.

Alkaline phosphatase labelled donkey anti-mouse IgG was purchased from Guildhay Antisera Ltd.

The phage lambda vector gt11 and Gigapack were obtained from NBL Enzymes Ltd.

M13mp9 DNA (RF) was obtained from New England Biolabs.

The plasmid vectors pUC13 and pKK232-8 were purchased from Pharmacia Biotechnology Group.

Horseradish peroxidase conjugated goat anti-rabbit IgG (H+L) was purchased from Miles Scientific.
Anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate; anti-human IgG (gamma-chain specific) alkaline phosphatase conjugate and anti-rabbit IgG (whole molecule) biotin conjugate were purchased from Sigma.

Rabbit anti-\textit{M. bovis} BCG antiserum and \textit{M. leprae} DNA were obtained from Dr. M.J. Colston.

The leprosy sera were obtained from a leprosarium in Spain.

5.7 Films

\begin{itemize}
  \item \textbf{Fuji:} X-ray film RX
  \item \textbf{Polaroid:} Positive/negative 4 x 5 land film type 665.
\end{itemize}
6.1 Maintenance of Strains

6.1.1 Escherichia coli strains

The *Escherichia coli* strains were streaked out on L-Agar plates and stored at 4°C. Working stocks were restreaked every month. For long term storage of strains, an equal volume of 2 x freezing buffer (see 1.4.5) was added to an overnight L-Broth culture of the strain to be stored.

6.1.2 Mycobacterium bovis BCG

This strain was maintained on Lowenstein-Jensen slopes. Cells were streaked over the entire surface of the Lowenstein-Jensen media and the slope was then incubated at 37°C for c. 3 weeks. Inoculated slopes were stored at 4°C.
6.2 Preparation of Chromosomal DNA

6.2.1 Isolation of Mycobacterium bovis BCG Chromosomal DNA

*M. bovis* BCG cell cultures were grown for 3 weeks, with rigorous shaking at 37°C, in Sauton media. Four methods were compared for the isolation of chromosomal DNA from large-scale cell cultures. Each method was slightly adapted from the previous one.

**Method I** (adapted from Marmur, 1961)

**Reagents:**

- TES: 10mM Tris-HCl pH 8, 100mM NaCl, 1mM Na₂EDTA
- TE: 10mM Tris-HCl pH 8, 1mM Na₂EDTA
- RNase A: 1mg/ml
- Alkaline/SDS: 0.3M NaOH, 10% SDS
- Phenol/TE
- Chloroform
- Unbuffered sodium acetate: 3M sodium acetate
- Ethanol

**Procedure**

Following 3 weeks growth at 37°C the *M. bovis* BCG cells were harvested by centrifugation (MSE-High Speed 18) at 10000 rpm for 15
minutes. The supernatant was discarded and the cell pellet was resuspended in TES buffer. Lysosyme was added to a final concentration of 1mg/ml, RNaseA was added to a final concentration of 50μg/ml and then the cell suspension was incubated at 37°C for 60 minutes. Alkaline SDS was added to the mixture to produce a final SDS concentration of 1%, and the mixture was then incubated at 65°C for 30 minutes. An equal volume of phenol/TE was added to the mixture, which was then vortexed. The two phases were separated by centrifugation (MSE chilspin) at 4000 rpm for 5 minutes. The aqueous phase (upper) was removed and vortexed with an equal volume of phenol/TE and chloroform. Separation of the two phases was again by centrifugation at 4000 rpm for 5 minutes. Once again the aqueous phase was removed but this time extracted with an equal volume of chloroform. Following separation of the two phases by centrifugation and recovery of the aqueous phase as before, 400μl volumes of the aqueous phase were aliquoted into 1.6ml microfuge tubes. A 0.1 volume of unbuffered sodium acetate and 2.5 volumes of ethanol were added to each microfuge tube and mixed by inversion. The chromosomal DNA was precipitated on ice for 20 minutes and recovered by centrifugation in an Eppendorf centrifuge for 10 minutes (at 4°C). The supernatant was discarded and the DNA pellet was resuspended in TE buffer, then stored at 4°C.

Method II (adapted from Rastogi et al., 1983)

Reagents:

20% Glycine

TES : 10mM Tris-HCl pH 8, 100mM NaCl, 1mM Na₂ EDTA

TE : 10mM Tris-HCl pH 8, 1mM Na₂ EDTA
RNaseA : 1mg/ml
Alkaline/SDS : 0.3M NaOH, 10% SDS
Phenol/TE
Chloroform
Unbuffered sodium acetate : 3M sodium acetate
Ethanol

Procedure

12 hours prior to commencing extraction of the chromosomal DNA, glycine was added to the cell culture, to a final concentration of 1%. Following a further 12 hours growth at 37°C, the M. bovis BCG cells were harvested by centrifugation and chromosomal DNA was extracted according to Method I.

Method III (adapted from Kieser, 1984)

Reagents:

20% Glycine
Lysosyme solution : Lysosyme (2mg/ml) in 0.3M sucrose, 10mM Tris-
HCl pH 8, 1mM Na₂ EDTA
RNaseA : 1mg/ml
Alkaline/SDS : 0.3M NaOH, 10% SDS
Proteinase K : 1mg/ml
Acid phenol/chloroform : 5g phenol (Analar), 5ml chloroform
1ml distilled water
Unbuffered sodium acetate: 3M sodium acetate

Ethanol

**Procedure**

Glycine was added to the cell culture (to a final concentration of 1%), 12 hours prior to commencing extraction of the chromosomal DNA. Following a further 12 hours growth at 37°C, the *M. bovis* BCG cells were harvested by centrifugation at 10000 rpm for 15 minutes. The pelleted cells were resuspended in a total volume of 3ml Lysosyme solution, containing 50μg/ml RNase A, and then incubated at 37°C for 3 hours. Following incubation, alkaline SDS was added to a final concentration of 1% and the cell suspension was then vortexed vigorously for 1 minute. Proteinase K was added to a final concentration of 50μg/ml and the mixture was incubated at 65°C for 15 minutes. An equal volume of acid phenol/chloroform was added to the mixture, which was then vortexed vigorously prior to centrifugation at 4000 rpm for 5 minutes. Centrifugation separated the two phases and the aqueous phase was recovered. The aqueous phase was vortexed together with an equal volume of chloroform and the two phases were separated once more by centrifugation at 4000 rpm for 5 minutes. The recovered aqueous phase was aliquoted into 400μl volumes, to each of which were added a 0.1 volume of unbuffered sodium acetate and 2.5 volumes of ethanol. The chromosomal DNA was precipitated on ice for 20 minutes, then recovered by centrifugation in an Eppendorf centrifuge. The supernatant was discarded and the DNA pellet was resuspended in TE buffer, then stored at 4°C.
Method IV (adapted from Hopwood et al., 1985)

Reagents:

20% Glycine

Lysosome solution: Lysosome (2mg/ml) in 0.3X sucrose, 25mM Tris-HCl pH 8, 25mM Na₂ EDTA

RNaseA: 1mg/ml

2X Kirby mixture: 2% sodium tri-isopropynaphthalene sulphonate, 12% sodium 4-amino salicylate, 100mM Tris-HCl pH 8, 6% phenol (Analar)

Phenol/TE

Chloroform

Unbuffered sodium acetate: 3M sodium acetate

Ethanol

Procedure

Once more, 12 hours prior to harvesting the cell culture, glycine was added to a final concentration of 1%. The M. bovis BCG cells were centrifuged at 10000 rpm for 15 minutes and the pelleted cells were resuspended in a total volume of 5ml lysosome solution, containing 50μg/ml RNaseA. Following incubation at 37°C for 60 minutes, 300μl volumes of the mixture were aliquoted into 1.6ml microfuge tubes, together with an equal volume of the 2X Kirby mixture (300μl), then vortexed vigorously for 1 minute. 300μl of phenol/TE and 300μl of chloroform were added to each tube and the contents were mixed by
vortexing. The two phases were separated by spinning the tubes for 2 minutes in an Eppendorf centrifuge. The aqueous phase was recovered and extracted with phenol/TE and chloroform a second time. Each aqueous phase was recovered and transferred into a fresh microfuge tube containing a 0.1 volume of unbuffered sodium acetate and 2.5 volumes of ethanol. The contents were mixed by inversion, then the tubes were incubated on ice for 20 minutes. The chromosomal DNA was pelleted by centrifugation in an Eppendorf centrifuge for 10 minutes (4°C) and the pellet redissolved in TE buffer.
6.2.2 Isolation of Chromosomal DNA from Escherichia coli strains

Reagents:

- Lysosyme solution: Lysosyme (1mg/ml) in TES buffer
- RNase: 1mg/ml
- Alkaline/SDS: 0.3M NaOH, 10% SDS
- Proteinase K: 1mg/ml
- Phenol/TE
- Chloroform
- Unbuffered sodium acetate: 3M Sodium Acetate
- Ethanol

Procedure

The strain under study was inoculated into L-Broth and grown, shaking at 37°C overnight. The cells were harvested by centrifugation at 10000 rpm for 15 minutes, then resuspended in the lysosyme solution, containing 50μg/ml RNaseA. The cell suspension was incubated at 37°C for 60 minutes, then Proteinase K (50μg/ml) and alkaline SDS (1%) were added to the cell suspension and the mixture was incubated at 65°C for 15 minutes. Equal volumes each of phenol/TE and chloroform were added to the mixture and mixed by vortexing. The two phases were separated by centrifugation at 4000 rpm for 5 minutes and the aqueous phase was recovered. The aqueous phase was then extracted with an equal volume of chloroform and once more recovered following centrifugation at 4000 rpm for 5 minutes. 400μl volumes of the extracted aqueous phase were
aliquoted into 1.6ml microfuge tubes, containing 0.1 volumes of unbuffered sodium acetate and 2.5 volumes of ethanol. The samples were mixed by inversion and the tubes were then incubated on ice for 20 minutes, to precipitate the DNA. Centrifugation in an Eppendorf centrifuge, for 10 minutes (at 4°C) pelleted the DNA, which was then resuspended in TE buffer and stored at 4°C.
6.3 Preparation of Plasmid DNA

6.3.1 Large-Scale Isolation of Plasmid DNA from E. coli strains

(extensively adapted from the method of Clewell and Helinski, 1969)

This procedure was used routinely to isolate plasmid DNA from E. coli strains and involves four basic steps: growth of the bacteria; amplification of the plasmid; harvesting and lysis of the bacteria and isolation and purification of the plasmid DNA.

Reagents:

L-Broth
Chloramphenicol
Sucrose solution: 25% sucrose in 50mM Tris-HCl pH 8
Lysosome solution: Lysosome (20mg/ml) in 0.25M Na₂ EDTA pH 8
0.25M Na₂ EDTA
Brij/Doc solution: 1% Brij 58, 0.4% SDS in 10mM Tris-HCl pH 8, 1mM Na₂ EDTA
Ethidium bromide: 20mg/ml
Caesium chloride
Isopropanol (saturated with a CsCl solution)
TES: 10mM Tris-HCl pH 8, 100mM NaCl, 1mM Na₂ EDTA
**Procedure**

A 10ml L-Broth starter culture (containing antibiotics where appropriate) of the strain under study, was incubated with shaking, at 37°C overnight. A 4ml aliquot of this starter culture was used to inoculate 150ml of pre-warmed L-broth (again containing antibiotics where appropriate), which was then incubated at 37°C with shaking for c. 3 hours, until the optical density of the culture at 650nm was 0.5. Solid chloramphenicol was added to the cell culture to obtain a final concentration of 150μg/ml. The culture was then reincubated at 37°C and shaken for a further 4-18 hours.

The cells were harvested by centrifugation (MSE-High Speed 18) at 6000 rpm (4°C) for 15 minutes and then resuspended in 2.6ml of the sucrose solution (ice-cold). The cell suspension was placed on ice and 0.4ml of the freshly prepared lysosyme solution was added. The suspension was swirled intermittently for 5 minutes (on ice) then 2.6ml of 0.25M Na₂EDTA was added and swirling continued intermittently for a further 5 minutes. Next, 4ml of the Brij/Doc solution was added and the suspension was mixed well by rapidly drawing it up and expelling it through a 10ml pipette. The mixture was incubated on ice for 20-30 minutes to obtain complete lysis of the cells.

The mixture was centrifuged at 15000 rpm (4°C) for 45 minutes and the cell debris and chromosomal DNA were pelleted. The supernatant was carefully transferred to a fresh tube, to which solid CsCl was added at a rate of 0.95g per ml supernatant. Ethidium bromide was then added...
to a final concentration of 500μg/ml. The resulting solution was then
distributed into Quick-Seal ultracentrifuge tubes, which were then
sealed and finally loaded into a 75Ti rotor and spun at 45000 rpm (20°C)
for 60 hours in a Beckman L8-M ultracentrifuge.

The DNA bands were viewed with long wave U.V. illumination. The
plasmid DNA band was removed by first puncturing the top of the tube,
then puncturing the side of the tube, just below the plasmid band, with
a 21G needle then finally drawing off the plasmid band with a 1ml
syringe. The ethidium bromide was removed from the plasmid DNA by three
extractions with an equal volume of the isopropanol/saturated CsCl
solution. The plasmid DNA solution was then dialysed against 4 x 500ml
volumes of TES buffer. The plasmid DNA was finally ethanol
precipitated, centrifuged and then resuspended in TE buffer.

6.3.2 Small-Scale Isolation of Plasmid DNA from E.coli strains
(Alkaline-lysis procedure described by Kieser, 1984)

This procedure was routinely used to isolate recombinant plasmid
DNA.

Reagents:

Lysosyme solution : Lysosyme (2mg/ml) in 0.3M sucrose, 25mM Tris-
HCl pH 8, 25mM Na2 EDTA
RNaseA : 1mg/ml
Alkaline/SDS : 0.3M NaOH, 2% SDS

- 86 -
Acid phenol/chloroform: 5g Phenol (Analar), 5ml chloroform (Analar), 1ml distilled water

Unbuffered sodium acetate: 3M sodium acetate
Isopropanol
100mM spermine-HCl
Salt solution: 0.3M sodium acetate, 10mM Magnesium chloride
Ethanol
TE: 10mM Tris-Cl pH 8, 1mM Na₂EDTA

Procedure

A 5ml L-Broth culture (containing antibiotics where appropriate) of the strain under study, was incubated with shaking at 37°C overnight. The cells were harvested by centrifugation at 4000 rpm (4°C) for 10 minutes. The pelleted cells were then resuspended in a 500μl volume of the lysosome solution, containing 50μg/ml RNaseA. The cell suspension was transferred into a 1.6ml microfuge tube and then incubated on ice for 30 minutes. Following incubation, the cell suspension was gently mixed by inversion, then 250μl of the alkaline SDS solution was added and the mixture was vortexed vigorously prior to incubation at 70°C for 15 minutes. The mixture was then allowed to cool to room temperature. 80μl of the acid phenol/chloroform solution was added to the mixture, which was then vortexed. The two phases were separated following spinning in an Eppendorf centrifuge for 2 minutes. The aqueous phase was removed and transferred into a fresh microfuge tube containing 70μl of unbuffered sodium acetate and 700μl isopropanol. The solutions were mixed by inversion, then incubated at room temperature for 5 minutes.
The DNA was pelleted by centrifugation, then resuspended in 500μl of TE. 25μl of the spermine-HCl solution was added, the solutions were mixed then incubated at room temperature for 5 minutes. The DNA was re-pelleted by centrifugation, then resuspended in 300μl 0.3M sodium acetate, 10mM MgCl₂. 700μl of ethanol was added and the sample was mixed by inversion, then incubated on ice for 20 minutes to precipitate the DNA. The DNA was pelleted once more by centrifugation (4°C, 10 minutes), and the pellet was redissolved in TE buffer.
6.4 Preparation of Phage Lambda DNA

6.4.1 Large-Scale Isolation of Phage Lambda DNA from Escherichia coli cultures

Reagents:
- L-Broth
- 1M MgSO₄
- Chloroform
- NaCl
- DNaseI
- RNaseA
- Polyethylene glycol (PEG) 6000

Caesium chloride solutions: (1) density 1.7g/ml; 97g CsCl in a total volume of 100ml SM buffer
(2) density 1.5g/ml; 67g CsCl in a total volume of 100ml SM buffer
(3) density 1.3g/ml; 40g CsCl in a total volume of 100ml SM buffer

TE : 10mM Tris-HCl pH 8.0, 1mM Na₂EDTA
10% SDS
0.5M Na₂EDTA

Procedure

A 10ml L-Broth, overnight culture of an appropriate E. coli strain
(NM539 was used for phage lambda EMBL4 and Y1090 for phage lambda gtII)
was prepared then used to inoculate a 500ml volume of pre-warmed L-
Broth, in a 5 litre flask. The culture was then incubated at 37°C with
shaking for c. 3 hours, until the optical density at 650nm was 0.5.
MgSO₄ was added to a final concentration of 10mM, together with
c.10⁹ p.f.u. of the appropriate phage stock and the culture was re-
incubated at 37°C and shaken for a further 2-3 hours. To aid cell
lysis, 1ml of chloroform was added to the culture, which was shaken at
37°C for a further 15 minutes. NaCl (20g) was added to the culture,
 together with DNaseI and RNaseA (each was added to a final concentration
of 1µg/ml) and the mixture was incubated at room temperature for 60
minutes, prior to centrifugation (MSE-High Speed 18) at 8000 rpm for 10
minutes at 4°C. The pelleted cell debris was discarded, 50g of
polyethylene glycol 6000 was dissolved in the supernatant and the
mixture was incubated at 4°C overnight.

Following overnight incubation, the mixture was centrifuged at
8000 rpm for 10 minutes at 4°C, the supernatant was discarded and the
phage pellet was gently resuspended in 10ml of SM buffer. The phage
suspension was then carefully layered onto a CsCl step-gradient which
had been prepared in a Quick-Seal ultracentrifuge tube as follows: 1.5ml
of CsCl solution 1 was pipetted into the ultracentrifuge tube; 2ml of
CsCl solution 2 was then carefully layered on top of the first solution,
without disturbing it and finally 2ml of CsCl solution 3 was carefully
layered on top of the second solution. Step-gradients were centrifuged
at 38000 rpm for 2 hours at 15°C and the turbid phage band was collected
by puncturing the tube with a 21G needle (just below the phage band) and
drawing off the band with a 1ml syringe. The collected material was
dialysed against 4 x 500ml volumes of TE buffer, to remove the caesium chloride.

Phage lambda DNA was extracted from the phage suspension by incubating the suspension with 0.1% SDS and 5mM Na₂EDTA at 68°C for 15 minutes and then subjecting the mixture to: (1) 2 x phenol/chloroform extractions and (2) 2 x chloroform extractions. The aqueous phase was then ethanol precipitated on ice for 20 minutes and the phage lambda DNA was finally recovered by centrifugation.

6.4.2 Small-Scale Isolation of Phage Lambda DNA from Plate Stocks

This procedure was routinely used to isolate recombinant phage lambda DNA.

Reagents:
L-Agar plates (243mm x 243mm)
SM buffer
1mg/ml DNaseI
1mg/ml RNaseA
NaCl
Polyethylene glycol 6000
10% SDS
0.5M Na₂EDTA
Phenol/chloroform
Chloroform
TE
Procedure

A 1.6ml L-Broth, overnight culture of the appropriate E. coli plating cells (NM539 for EMEL4 and Y1090 for gt11) was mixed with c. 10⁷ phage particles and incubated at 37°C for 15 minutes. The mixture was then added to a 25ml volume of molten T-top agar (supplemented with 10mM MgSO₄), mixed carefully to prevent the formation of air-bubbles and poured immediately onto a 243mm x 243mm L-Agar plate. When the overlay had set, the plate was incubated at 37°C overnight. An amplified phage stock was subsequently prepared by overlaying the plate with a 40ml volume of SM buffer and then incubating the plate at 4°C overnight.

The amplified phage stock was recovered from the L-Agar plate and centrifuged at 8000 rpm for 10 minutes at 4°C to pellet cell debris. DNaseI and RNaseA were then each added to the supernatant, to a final concentration of 1μg/ml and this mixture was incubated at room temperature for 60 minutes. NaCl was added to a final concentration of 1M and the mixture was incubated on ice for 60 minutes. The mixture was then centrifuged at 8000 rpm for 10 minutes at 4°C and the pellet was discarded. Polyethylene glycol 6000 was dissolved in the supernatant, to a final concentration of 10% and the mixture was incubated on ice for 60 minutes, to precipitate the phage particles. Following centrifugation at 8000 rpm for 10 minutes at 4°C, the supernatant was discarded and the phage pellet was resuspended in 2ml of TE buffer.

A 20μl aliquot of 10% SDS was added to the phage suspension, together with a 20μl aliquot of 0.5M Na₂EDTA and the phage suspension
was then incubated at 68°C for 15 minutes. Following incubation, the phage suspension was cooled to room temperature and then extracted twice with phenol/chloroform and then twice with chloroform alone. Finally the recovered aqueous phase was ethanol precipitated on ice for 20 minutes and the phage lambda DNA pelleted by centrifugation.
6.5 **In Vitro Manipulation of DNA**

6.5.1 **Ethanol Precipitation of DNA**

Precipitation of DNA was achieved by aliquoting 400 µl volumes of the DNA solution into 1.6 ml microfuge tubes containing a 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. This mixture was inverted several times, then incubated on ice for 20 minutes to precipitate the DNA. The DNA was then pelleted by spinning in an Eppendorf centrifuge (4°C) for 10 minutes. The supernatant was discarded and the DNA pellet was washed with 500 µl of 70% ethanol (cold). The DNA was pelleted as before, the supernatant was discarded and then the pellet was dried under vacuum for 5 minutes. The DNA pellet was then resuspended in TE buffer.

6.5.2 **Quantitation of DNA**

The DNA concentration (and protein concentration) was determined by spectrophotometrically measuring the amount of UV radiation absorbed by the bases (Maniatis et al., 1982).

**Procedure**

DNA preparations were diluted 10 or 20-fold in distilled water. The optical density at 260 nm and 280 nm was then measured using a Pye Unicam model PU8820 UV/vis spectrophotometer and a 0.5 ml quartz cuvette. The ratio of the readings obtained at 260 nm and 280 nm (OD260/OD280)
provides an estimate of the purity of the DNA solution, with OD260/OD280 ratios greater than 1.7 indicating DNA preparations of high purity with very little protein contamination. An optical density of 1.0 at 260nm is equivalent to a DNA concentration of 50μg/ml. Consequently DNA concentrations can be determined from the following formula:

\[
\text{DNA concentration} = (\text{OD}_{260} \times 50 \times \text{Dilution}) \ \mu\text{g/ml.}
\]

6.5.3 Endonuclease Digestion of DNA

Single Restriction digests

Typical restriction digests, with one endonuclease were set up as follows:

10μl DNA (0.1-1.0μg)
2μl 10X Digestion buffer
1μl Restriction Endonuclease
7μl distilled water

Each restriction endonuclease has a particular buffer composition which optimises the restriction digest. These buffers were prepared according to the manufacturers instructions. Digests were incubated at the appropriate temperature (according to the manufacturers instructions) for 60 minutes. Digests were stopped by heating to 65°C for 10 minutes.
Double Restriction Digests

Digestion with the first endonuclease was carried out as described above. Following digestion for 60 minutes, the reaction was stopped by heating to 65°C for 10 minutes. If the second enzyme had the same buffer requirements as the first enzyme, then 1μl of the second restriction endonuclease was simply added to the reaction mixture which was then incubated at the appropriate temperature for 60 minutes. If however the buffer requirement for the second endonuclease was different from that of the first endonuclease, then adjustments to the salt concentration had to be carried out prior to addition of 1μl of second restriction endonuclease. Following 60 minutes digestion the reaction mixture was heated to 65°C for 10 minutes to inactivate the endonuclease.

Partial Endonuclease Digestion

Chromosomal DNA aliquots were only partially digested with restriction endonucleases, in order to produce suitably sized restriction fragments for cloning (i.e. fragments ranging in size from 1-6Kb for cloning into plasmid vectors and fragments 9-23Kb in size for cloning into lambda phage vectors). Digests were prepared according to the method described earlier (see single endonuclease digests) however incubation reaction times were only 15-30 minutes, and the degree of digestion was determined by agarose gel electrophoresis (see section 6.5.5).
6.5.4 Dephosphorylation of DNA

The dephosphorylation reaction removes terminal 5' phosphate groups, exposed following digestion of DNA with restriction endonucleases. In particular, digested vector DNA was dephosphorylated in order to prevent self-ligation of vector DNA molecules during ligation reactions with insert DNA, thus encouraging the formation of recombinant molecules. Two enzymes can be used to achieve dephosphorylation: bacterial alkaline phosphatase (BAP) and calf intestinal alkaline phosphatase (CIAP) (Chaconas and Van de Sande, 1980). The latter enzyme has the considerable advantage in that it can be completely inactivated by heating to 68°C, consequently CIAP was the enzyme preferred during the present study.

Procedure

Following restriction endonuclease digestion, linearised vector DNA was extracted once with phenol/chloroform and then ethanol precipitated. The DNA pellet was resuspended in TE buffer and 0.1 unit of CIAP was added per microgram of DNA. The reaction mixture was then incubated at 37°C for 30 minutes. The dephosphorylation reaction was terminated by heating to 70°C for 30 minutes, the reaction mixture was then subjected to two phenol/chloroform extractions and finally ethanol precipitated. The DNA was pelleted then resuspended in TE buffer.
6.5.5 Agarose Gel Electrophoresis

The mobility of DNA in a gel is determined principally by its size: smaller molecules run faster. A 1% agarose gel will allow reasonable separation of fragments 1-10Kb in size; at this concentration larger fragments will tend not to separate, while smaller fragments, although widely separated, will be rather diffuse and some may even have run off the gel. To separate larger fragments for analysis, agarose concentrations <1% should be used, whilst agarose concentrations >1% will allow easier analysis of smaller fragments. In this study horizontal agarose gel were commonly used: (1) in order to analyse the completeness or otherwise of restriction endonuclease digests and (2) to size fractionate digested DNA.

Reagents:

Agarose

TBE Running Buffer: 89mM Tris-base, 89mM boric acid, 2mM Na₂EDTA

Procedure

The gel apparatus principally used was a horizontal, Minigel (Uniscience) tank, requiring 30ml agarose. The agarose concentration most commonly used was 1%, prepared by weighing out 0.3g agarose into a conical flask containing 30ml of running buffer. This solution was heated in a boiling water bath until the agarose dissolved, then cooled slightly prior to pouring. The horizontal gel tank was prepared by
inserting spacers into their appropriate positions. Once the gel was poured a comb was inserted into the molten agarose. The gel was then left to set at room temperature for 30 minutes. The comb and spacers were carefully removed and running buffer was poured over the gel, covering it by c.2mm. The wells formed by the comb were capable of holding 15µl volumes: 10µl DNA samples and 5µl borate loading buffer (50% glycerol, 5% SDS, 0.25% bromophenol blue). DNA samples were electrophoresed at the appropriate voltage until the marker dye had run almost to the bottom of the gel, then electrophoresis was stopped. The gel was stained with 0.5µg/ml ethidium bromide for 5 minutes, then destained with distilled water. The DNA bands within the gel were visualised via long wave UV illumination provided by a transilluminator (Ultra-Violet Products Inc.). The gel was photographed when required.

6.5.6 Recovery of DNA Fragments from Horizontal Agarose Gels (Young et al., 1985a)

DNA samples were loaded and electrophoresed through horizontal agarose gels containing 0.5µg/ml ethidium bromide (to allow rapid visualisation of the DNA fragments undergoing size-fractionation). A piece of NA45 filter paper was inserted into a slit, cut just in front of the fragment(s) to be recovered, and electroelution was continued until the appropriate fragment(s) had bound completely to the NA45 filter paper. The filter paper was removed from the gel, rinsed in TE buffer (pH 8), then incubated at 70°C for 2 hours in a 1.6ml microfuge tube containing 0.4ml IM NaCl, 0.05M arginine (free base), to elute the DNA from the filter paper. After elution of the DNA fragment(s), the
filter paper was removed from the microfuge tube and the DNA fragment(s) were ethanol precipitated on ice for 20 minutes (section 6.5.1). DNA pellets were resuspended in TE buffer.
5.6 Preparation of Plasmid Genomic DNA Libraries

5.6.1 Ligation of Plasmid Vector DNA and Insert DNA

Two plasmid vectors, pBR322 (Bolivar et al., 1977) and pUC13 (Vieira and Messing, 1982) were employed in the preparation of four mycobacterial genomic DNA libraries. The vector DNA's were completely digested with the restriction enzyme BamHI (as described in section 6.5.3) and were then treated with calf intestinal alkaline phosphatase (CIAP), to remove the terminal 5' phosphate groups (exposed as a consequence of the digestion reactions), to prevent self-ligation of the vector DNA molecules during the subsequent ligation reactions. Aliquots of the mycobacterial DNA's (M. bovis BCG and M. leprae DNA) were partially digested with either BamHI or Sau3A and the resultant restriction fragments were ligated into the BamHI-digested, CIAP-treated vector molecules, as described below and each of the ligation mixtures was then incubated at 12°C overnight.

1. pBR322/M. bovis BCG Library

2μg pBR322 DNA, BamHI-digested, CIAP-treated + 3μg M. bovis BCG DNA, BamHI-digested + 10μl 5mM ATP + 10μl 10X Ligation buffer + 15μl distilled water + 5 units T4-DNA Ligase.

2. pBR322/M. leprae Library 1.

150ng pBR322, BamHI-digested, CIAP-treated + 100ng M. leprae DNA, BamHI-digested + 2μl 5mM ATP + 2μl 10X Ligation buffer + 1 unit T4-DNA Ligase.
3. **pBR322/M.leprae Library 2.**

150ng pBR322, BamHI-digested, CIAP-treated + 100ng *M.leprae* DNA, Sau3A-digested + 2μl 5mM ATP + 2μl 10X Ligation buffer + 1 unit T4-DNA Ligase.

4. **pUC13/M.leprae Library**

150ng pUC13, BamHI-digested, CIAP-treated + 100ng *M.leprae* DNA, Sau3A-digested + 2μl 5mM ATP + 2μl 10X Ligation buffer + 1 unit T4-DNA Ligase.

6.6.2 **Transformation of Escherichia coli Cells** (Kushner, 1978)

Generally the products of ligation reactions are highly heterogeneous mixtures of recombinant molecules (foreign DNA molecules ligated to plasmid DNA molecules) and non-recombinant molecules (parental plasmids) and transformation is the method by which these ligation products are introduced into competent *E.coli* cells. Furthermore, the conditions of transformation are such that, only a single plasmid molecule enters a bacterial cell and the resultant transformants, when plated out onto solid media, will produce colonies, each of which contains a homogeneous population of cloned plasmid molecules.

**Reagents:**

Solution 1: 10mM MOPS; 10mM RbCl pH 7.0

Solution 2: 100mM MOPS; 50mM CaCl₂; 10mM RbCl pH 6.5
Procedure

A 10ml L-Broth culture of the appropriate E. coli transformation strain (HB101 for pBR322; JM103 for pUC13) was grown at 37°C overnight. A 0.1ml aliquot of the overnight culture was then used to inoculate a 20ml volume of pre-warmed L-Broth and this culture was then grown, with vigorous shaking, at 37°C until the optical density of the culture was approximately 0.15 at 650nm.

Aliquots (1.5ml) of the culture were then pelleted by centrifugation in an Eppendorf centrifuge for 5 minutes. The supernatants were discarded and the pellets were resuspended in 0.5ml aliquots of ice-cold Solution 1. The samples were immediately repelleted and the pellets were resuspended in 0.5ml aliquots of ice-cold Solution 2 and then incubated on ice for 60 minutes. The samples were repelleted and the pellets resuspended in a 0.2ml aliquot of ice-cold Solution 2. (The cells are competent at this stage.)

An aliquot (10–20μl) of ligated DNA (section 6.6.1) was added to each 0.2ml volume of competent E. coli cells and the samples were incubated on ice for 30–45 minutes, with occasional shaking. The samples were heated at 45°C for 30 seconds and then immediately re-incubated on ice. A 2ml volume of pre-warmed L-Broth was added to each sample, which was then incubated at 37°C for 60 minutes. Following incubation, the samples were pelleted and the pellets were resuspended in 0.1ml volumes of L-Broth. Each of the samples were plated out onto L-Agar plates containing the appropriate antibiotics; to allow the
selection of transformants from non-transformants (E. coli strains HB101 and JM103 are sensitive to ampicillin, but become resistant when transformed with the plasmids pBR322 and pUC13, respectively). The L-Agar plates were then incubated at 37°C overnight.

6.6.3 Amplification and Storage of Transformants

Plates containing plasmid gene library transformants (bacterial colonies) were overlaid with 5ml volumes of L-Broth. A glass-spreader was then used to remove the colonies from the surface of the plates, producing cell suspensions. The cell suspensions were pooled, diluted with an equal volume of 2X Freezing buffer (section 5.4), then stored at -70°C until required.

Alternatively, individual transformants were removed from the plates with the aid of toothpicks, into separate aliquots of L-Broth. These were then incubated at 37°C overnight, to produce amplified stocks of each clone, then diluted with an equal volume of the 2X Freezing buffer and finally stored at -70°C.
6.7 Preparation of Phage Lambda Genomic DNA Libraries

Two phage lambda DNA cloning vectors, EMBL4 (Frischauf et al., 1983) and gtII (Young and Davis, 1983 a; b) were employed to prepare
*M. bovis* BCG and *M. leprae* genomic DNA libraries. BamHI-digested EMBL4
DNA and EcoRI-digested gtII DNA were purchased from their respective
manufacturers (Amersham International and NBL Enzymes Ltd) and without
further treatment were ligated with mycobacterial DNA, as described
below. The recombinant DNA molecules produced were then packaged in
vitro using extracts from a Gigapack Packaging Kit (NBL). The extracts
used for packaging are prepared from phage lambda derivatives, each of
which is defective for a specific component required for packaging and
by using two extracts, each complementing the others defect, the
complete packaging system is provided. Recombinant DNA molecules, once
packaged, can then be introduced into the appropriate *E. coli* host cell
by the normal process of phage infection, i.e. phage adsorption and DNA
injection. The phage particles produced as a result of the packaging
reaction constitute the basic genomic library.

6.7.1 Production of Recombinant EMBL4/*M. bovis* BCG Molecules

*M. bovis* BCG DNA (2μg) was incubated with 10 units of restriction
enzyme BamHI at 37°C for 20 minutes. The digest was then
electrophoresed through a 1% agarose gel to size fractionate the *M. bovis*
BCG, restriction fragments. Partial digestion was revealed to have
produced restriction fragments 1-20Kb in size. In order that viable
phage are produced (that may infect *E. coli* cells), DNA insert fragments
of 9-23Kb need to be cloned into the BamHI-digested EMBL4 cloning vector, consequently BamHI restriction fragments, greater than 9Kb in size, were recovered from the agarose gel in accordance with the procedure described in section 6.5.6.

The following ligation reaction was subsequently prepared and incubated at 12°C overnight.

A: Ligation Reaction of *M. bovis* BCG DNA and Phage Lambda Vector EMBL4

1µg EMBL4, BamHI-digested + 200ng *M. bovis* BCG DNA, BamHI-digested + 1µl 5mM ATP + 1µl 10X Ligation buffer + 1 unit T4-DNA Ligase.

6.7.2 Production of Recombinant *gtll* Molecules (Huynh et al., 1985)

To ensure that all of the coding sequences in the genome of interest (i.e. *M. bovis* BCG or *M. leprae*) are expressed, requires the construction of a recombinant DNA library whose insert fragments are present in all orientations and translation frames. To generate large numbers of insert fragments whose endpoints occur at random throughout the genome, 2µg of *M. bovis* BCG DNA and 1µg of *M. leprae* DNA (each in a 100µl volume) were mechanically sheared by 225 passages through a 25-gauge needle. The sheared DNA's were then electrophoresed through a 1% agarose gel, revealing fragments of 1-15Kb in size. Only fragments up
to 7Kb in size were recovered from the gel, as ligating insert larger than 7Kb into gtII produces non-viable recombinant molecules.

The EcoRI restriction sites (within the sheared DNA fragments) were methylated by resuspending the sheared DNA fragments (1-7Kb M.bovis BCG and M.leprae) in 20μl of EcoRI methylase buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 5mM DTT, 10μM S-adenosyl-L-methionine) and then incubating the mixtures at 37°C for 15 minutes, in the presence of 20 units of EcoRI methylase. The methylation reaction was terminated by incubating the mixture at 70°C for 10 minutes.

To blunt-end the methylated DNA fragments, 2.5μl of 100mM MgCl₂; 2.5μl of 0.2mM dNTP's (i.e. dATP, dTTP, dCTP, dGTP) and 5 units of T4-DNA Polymerase were added to the 20μl reaction mixtures and these were then incubated at room temperature for 10 minutes. Following incubation, EDTA was added to a final concentration of 15mM, the mixtures were then extracted with phenol/chloroform and the DNA's were then precipitated with ethanol.

The DNA pellets were resuspended in 6μl of TE buffer and the following ligation reactions were prepared and incubated at 12°C for 4 hours:

A: **Ligation of Mycobacterial DNA and EcoRI Linkers**

6μl methylated, blunt-ended DNA (M.bovis BCG or M.leprae) + 1μl 10mM ATP + 1μl 10X Ligation buffer + 1 unit T4-DNA ligase + 1μl EcoRI linkers.
To remove excess EcoRI linkers and to generate EcoRI cohesive ends, 7 µl of distilled water and 2 µl of 10X EcoRI digestion buffer (10X High buffer) were added to the 10 µl ligation reactions described above and these were then incubated at 70°C for 10 minutes to destroy the ligase activity. 130 units of restriction enzyme EcoRI were then added to each mixture and these were incubated at 37°C for c. 1.5 hours. To separate the DNA fragments from the excess EcoRI linkers, the two digests were electrophoresed through a 1% agarose gel and the DNA fragments were subsequently recovered as described in section 6.5.6.

Once again, following ethanol precipitation, the DNA pellets were resuspended in 6 µl of TE buffer and the following ligation reactions were then prepared and incubated at 12°C overnight.

B: Ligation of Mycobacterial DNA and the Phage Lambda Vector gtII

1µg gtII, EcoRI-digested + 200ng mycobacterial DNA (M. bovis BCG or M. leprae) sheared, methylated, EcoRI linkers + 1 µl 10mM ATP + 1 µl 10X Ligation buffer + 1 unit T4-DNA ligase.

6.7.3 In Vitro Packaging

Reagents:

Gigapack Packing Kit: freeze-thaw extract and a sonic extract.

SM buffer: 0.2M NaCl, 1mM MgSO4.7H2O, 50mM Tris-HCl pH 7.5, 0.01% gelatin.
**Procedure**

A 4μl aliquot of recombinant DNA (EMBL4/M. bovis BCG DNA; gtII/M. bovis BCG or gtII/M. leprae) was added to a freeze-thaw extract and this mixture was incubated on ice. Immediately, 15μl of the sonic extract was added to the freeze-thaw + recombinant DNA mixture and this mixture was then incubated at 22°C for 2 hours. Following incubation, a 500μl aliquot of SM buffer and a 20μl aliquot of chloroform were added to the in vitro packaging mixture and the mixture was incubated at 4°C.

**6.7.4 Transfection of Escherichia coli Cells**

A 10ml L-Broth culture (supplemented with 10mM MgSO₄) of the appropriate E.coli host strain (i.e. E.coli NM539 for recombinant EMBL4 molecules and E.coli Y1088 for recombinant gtII molecules) was grown at 37°C overnight. The culture was then pelleted at 4000rpm for 5 minutes at 4°C and the cells were then resuspended in 2.5ml of 10mM MgSO₄.

Diluted aliquots of the EMBL4 packaging mixture were prepared with SM buffer, these were then mixed with 0.2ml aliquots of E.coli NM539 cells and incubated at 37°C for 15 minutes, to allow phage adsorption to occur. Each mixture was then added to a 3ml volume of molten T-top agar, which was then poured immediately onto a pre-warmed L-Agar plate. When the overlays had set, the plates were incubated at 37°C overnight. Following overnight incubation, the number of plaques per L-Agar plate were determined and the EMBL4/M.bovis BCG library size was calculated.
Diluted aliquots of the gtII packaging mixtures were prepared and mixed with 0.2ml aliquots of *E.coli* Y1088 cells. Following incubation at 37°C for 15 minutes, these samples were mixed with 3ml volumes of molten T-top agar and then poured onto L-Agar plates containing 10mM IPTG and 0.2% X-gal. These plates were incubated at 42°C overnight, when the overlays had set. Plating out aliquots of the gtII packaging mixtures (*M.bovis* BCG and *M.leprae*) on media containing IPTG and X-gal allowed determination of the percentage of recombinant phage molecules in each gtII library. Recombinant phage were identified as clear plaques and non-recombinant phage were blue.

6.7.5 Amplification of Phage Lambda Libraries

To amplify phage lambda libraries, 0.2ml aliquots of the appropriate *E.coli* host (*E.coli* NM539 for recombinant EMBL4 molecules and *E.coli* Y1088 for recombinant gtII molecules) were infected with 50μl aliquots of the appropriate in vitro packaged phage molecules. The cells + phage mixtures were then added to 3ml volumes of molten T-top agar and these were then immediately poured onto pre-warmed L-Agar plates. When the overlays had set the EMBL4 plates were incubated at 37°C overnight and the gtII plates were incubated at 42°C overnight.

Following overnight incubation, plate stocks were prepared by overlaying each of the plates with a 5ml volume of SM buffer and then incubating each of these plates at 4°C overnight. The SM buffer overlays were subsequently removed and pooled (where appropriate); DMSO was added to a final concentration of 7% and the amplified phage library stocks were then stored at -70°C.
6.8 Absorption of Primary Antibody Solutions

A polyclonal antiserum solution often contains anti-\textit{E. coli} antibodies and these can cause problems when the serum is used to screen gene libraries cloned in \textit{E. coli} hosts, through the binding of these antibodies to the natural antigens expressed by the \textit{E. coli} host, resulting in high background signals. These anti-\textit{E. coli} antibodies can be most effectively removed from the polyclonal antiserum solution by immobilising \textit{E. coli} lysates on a nitrocellulose filter and then incubating this filter with the antiserum solution.

Reagents:

\textbf{PBS} : 8g/litre NaCl; 0.2g/litre KCl; 0.2g/litre K$_2$PO$_4$; 1.15/litre Na$_2$HPO$_4$ pH 7.3.

\textbf{TBST} : 50mM Tris-HCl pH 7.5; 150mM NaCl; 0.05% Tween 20.

Procedure

A 250ml volume of L-Broth was inoculated with the appropriate \textit{E. coli} strain (HB101, Y1089, Y1090) and following overnight incubation at 37°C, the culture was harvested by centrifugation at 10 000 rpm for 15 minutes. The pelleted cells were then resuspended in a 25ml volume of PBS. The cells were then sonicated on ice (MSE sonicator : power = medium; amplitude = 3) and the resultant crude \textit{E. coli} lysate was then bound to a nitrocellulose filter by incubating the filter in the lysate solution for 5 minutes. Unbound lysate was removed from the filter.
following two, 1 minute washes of the filter in TBST. A 1:10 dilution of the antiserum solution was prepared in PBS and the crude lysate-bound filter was then incubated in the diluted antiserum solution for 30 minutes. This filter was then removed from the antiserum solution and replaced with a fresh, crude lysate-bound filter. Incubation of the antiserum solution with the second filter was again carried out for 30 minutes. The filter was then discarded and the absorbed antiserum solution was recovered and stored at -70°C until required.
6.9 In Situ Screening of Plasmid Gene Libraries (adapted from Engleberg et al., 1984)

Reagents:

- 90mm L-Agar plates
- Chloroform
- TBS: 50mM Tris-HCl pH 7.5, 150mM NaCl
- TBST: 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20
- BSA: Bovine serum albumin

Primary antibodies:

- E.coli HB101 absorbed rabbit anti-M.bovis BCG antiserum; mouse anti-M.leprae monoclonal antibodies; E.coli absorbed serum from a lepromatous leprosy patient.

Secondary antibodies:

- horseradish peroxidase conjugated goat anti-rabbit IgG (Miles);
- horseradish peroxidase conjugated rabbit anti-mouse IgG (Miles);
- alkaline phosphatase conjugated goat anti-human IgG (Sigma).

Colour development solutions:

- horseradish peroxidase colour development solution; alkaline phosphatase colour development solution.

Procedure

Diluted aliquots of the appropriate plasmid library (pBR322/M.bovis BCG, pBR322/M.leprae, pUC13/M.leprae) were prepared and plated out onto L-Agar plates containing 50μg/ml ampicillin (or
individual clones were stabbed onto plates with the aid of toothpicks), which were then incubated at 37°C overnight. Following overnight incubation, the colonies on the plates were overlaid with nitrocellulose filters (90mm disc; Schleicher and Schuell) and the plates + filters were re-incubated at 37°C for 5 minutes. The nitrocellulose filters were removed from the L-Agar plates (cells remained bound to the filters) and were placed colony side up on chloroform-soaked Whatman No. 1 filter papers for 5 minutes in order to lyse the bound cells (the plates were stored at 4°C until required). Following the chloroform treatment, the nitrocellulose filters were then air dried for c.15 minutes.

The air-dried filters were then incubated for 60 minutes in a solution of TBST containing 3% BSA, to block the non-specific protein binding sites on the filters. Following this treatment, the filters were incubated for 60 minutes in a solution of TBST + 3% BSA, containing the appropriate primary antibodies. Following incubation, the filters were washed twice (5 minutes each wash) in TBST, to remove unbound primary antibody. The filters were then incubated in a solution of TBST, containing the appropriate secondary antibodies, for 60 minutes. On their removal from the secondary antibody solution, the filters were washed in a solution of TBS for 60 minutes (with three changes of buffer). Finally, the filters were incubated in either (i) the horseradish peroxidase colour development solution: 0.05% 4-chloro-1-napthol, 0.015% hydrogen peroxide in a 5:1 solution of TBS-methanol or (ii) the alkaline phosphatase colour development solution: 0.033%
nitroblue tetrazolium (NBT); 0.016% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100mM Tris-HCl pH 9.5, 100mM NaCl, 5mM MgCl₂.

Positive signals were detected on the filters as blue/purple spots and the colonies responsible for these positive signals (i.e. clones expressing \textit{M.bovis} BCG or \textit{M.leprae} antigens) were identified by re-aligning the filters with the appropriate L-Agar plates. Positive colonies were subsequently recovered from the L-Agar plates, with the aid of toothpicks and were used to inoculate 2ml volumes of L-Broth (containing 50\(\mu\)g/ml ampicillin). Each of these 2ml samples was then incubated at 37°C overnight, to prepare amplified stocks.
6.10 In Situ Screening of Phage Lambda Gene Libraries

6.10.1 Screening Phage Lambda gtII Clones

Reagents:

L-Agar plates (243mm x 243mm)
10mM IPTG (isopropyl beta-D-thiogalactopyranoside)
TBS : 50mM Tris-HCl pH 7.5, 150mM NaCl
TBST : 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20
BSA : Bovine serum albumin

Primary antibodies:

- E.coli absorbed rabbit anti-M.bovis BCG antiserum; mouse anti-M.leprae monoclonal antibodies; serum from leprosy patients

Secondary antibodies:

- alkaline phosphatase conjugated goat anti-rabbit IgG; alkaline phosphatase conjugated donkey anti-mouse IgG; alkaline phosphatase conjugated goat anti-human IgG; biotin conjugated goat anti-rabbit IgG (and streptavidin-biotinylated horseradish peroxidase complex).

Colour development solution:

- alkaline phosphatase colour development solution; horseradish peroxidase colour development solution.
A 10ml L-Broth culture of *E. coli* strain Y1090 was prepared and incubated at 37°C overnight. Aliquots (1.6ml) of this overnight culture were then infected with aliquots of the appropriate gtII library (c.5 x 10^4 phage per plate) and the infections were incubated at 37°C for 15 minutes to allow adsorption of the phage to the cells to take place. Each infection mix was then added to a 25ml volume of molten T-top agar and poured immediately onto an L-Agar plate. When the overlays had set, the plates were incubated at 42°C. Following 3 hours incubation at 42°C the L-Agar plates were overlaid with nitrocellulose filters, which had previously been soaked in 10mM IPTG and the plates + filters were then incubated at 37°C for 3 hours.

Following incubation at 37°C, the nitrocellulose filters were removed from the L-Agar plates and incubated for 60 minutes in a solution of TBST containing 3% BSA, to saturate the non-specific protein binding sites on the nitrocellulose filters. The filters were then incubated for 60 minutes in a solution of TBST + 3% BSA, containing the appropriate primary antibodies. Following incubation, the filters were washed twice (5 minutes each wash) in TBST and were then incubated in a solution of TBST containing the appropriate secondary antibodies, for 60 minutes. On removal from the secondary antibody solution, the filters were washed in a solution of TBS for 60 minutes. (Those filters which had been incubated with biotin conjugated secondary antibodies were washed with TBST for 10 minutes and then incubated in a solution of TBST containing the streptavidin-biotinylated horseradish peroxidase complex.
for 60 minutes, then washed in a solution of TBS for 60 minutes.) The washed filters were then incubated in either (i) the alkaline phosphatase colour development solution or (ii) the horseradish peroxidase colour development solution (section 6.9).

Positive signals were detected as blue/purple doughnut shapes on the filters and the plaques responsible (i.e. clones expressing \textit{M. bovis} BCG or \textit{M. leprae} molecules) were identified by re-aligning the filters with the appropriate L-Agar plates. Whenever possible, positive plaques were discretely picked from the L-Agar plates (with the aid of toothpicks) into 50μl volumes of SM buffer, which were then incubated at 4°C to produce amplified phage stocks. When the density of plaques on the plate was too great to allow individual positive plaques to be selected, or when the identity of the positive plaque could not be pinpointed accurately, a number of plaques were scraped from the plate, in the region corresponding to the positive signal, and dispersed in 100μl volumes of SM buffer. These samples were then plaque-purified as described in section 6.11.

6.10.2 Screening Phage Lambda EMBL4 Clones

Reagents:

L-Agar plates (243mm x 243mm)

TBS : 50mM Tris-HCl pH 7.5, 150mM NaCl

TBST : 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20

BSA : Bovine serum albumin

Primary antibodies : \textit{E. coli} absorbed rabbit anti-\textit{M. bovis} BCG
antiserum.

Secondary antibodies: alkaline phosphatase conjugated goat anti-rabbit IgG.

Colour development solution: alkaline phosphatase colour development solution.

Procedure

A 10ml L-Broth culture of E.coli strain NM539 was prepared and incubated at 37°C overnight. Aliquots (1.6ml) of this overnight culture were infected with aliquots of the EMBL4/M.bovis BCG library (c.5 x 10⁴ phage per plate) and the infections were incubated at 37°C for 15 minutes to allow adsorption of the phage to the cells to occur. Each infection mix was then mixed with a 25ml volume of molten T-top agar, which was then poured immediately onto an L-Agar plate. When the overlays had set, the plates were incubated at 37°C for 7 hours. The plates were then overlaid with nitrocellulose filters and the plates + filters were incubated at 37°C for a further 3 hours.

The nitrocellulose filters were removed from the L-Agar plates and incubated in a solution of TBST containing 3% BSA, then incubated in a solution of TBST + 3% BSA containing the primary antibodies. Following incubation in this solution the filters were washed twice in TBST, then incubated in a solution of TBST containing the secondary antibodies. On their removal from the secondary antibody solution, the nitrocellulose filters were washed in TBS for 60 minutes, then incubated in the alkaline phosphatase colour development solution.
6.11 The Plaque-Purification of Individual Clones

Following the in situ screening of phage libraries (section 6.10) positive signals were detected as blue/purple doughnut shapes on nitrocellulose filters and the subsequent re-alignment of these nitrocellulose filters with the appropriate L-Agar plates resulted in the identification of the plaques responsible for these signals. When the plaque density on the L-Agar plate is low, the positive plaque can be removed discretely from the L-Agar plate with the aid of a toothpick and this material is then dispersed in a 50μl volume of SM buffer and incubated at 4°C overnight to produce a plaque-purified phage stock of the positive plaque.

Often however, the plaque density on the L-Agar plates is too high to allow the positive plaques to be removed discretely. In these situations a number of plaques are scraped from the plate, surrounding and including the positive plaque. This material, when dispersed in a 50μl volume of SM buffer, results in the production of a pre-plaque purified phage stock. Diluted aliquots of this phage stock are then prepared, mixed with 200μl volumes of E.coli cells (i.e. Y1090 cells for gtll clones and NW539 cells for EMBL4 clones) and then incubated at 37°C for 15 minutes to allow adsorption of the phage to the cells to occur. The infection mixtures are then mixed with 3ml volumes of molten T-top agar and plated out onto L-Agar plates. When the overlays have set: (1) plates containing gtll clones are incubated at (i) 42°C for 3-4 hours and then (ii) overlaid with IPTG-soaked nitrocellulose filters and incubated at 37°C for 3-4 hours and (2) plates containing EMBL4 clones
are incubated at (i) 37°C for 7 hours and then (ii) overlaid with nitrocellulose filters and incubated at 37°C for a further 3 hours.

Following their removal from the plates, the filters are incubated in the appropriate primary and secondary antibody solutions (as described previously) and are then incubated in the appropriate colour development solution. Development of the filters should reveal a number of positive signals on each filter and consequently it should then be possible to discretely remove a plaque, corresponding to a positive signal, from a plate with a low density of plaques. Material picked from the positive plaque is then dispersed in a 50μl volume of SM buffer, producing a plaque-purified phage stock of the expression positive clone. In situ screening of a nitrocellulose blot of a plaque-purified positive clone will result in the detection of positive signals to all 100% of the plaques screened.

An amplified phage stock of a plaque-purified expression positive clone is prepared by plating out E.coli cells with phage from the plaque-purified stock (i.e. Y1090 cells for gt11 clones and NM539 cells for EMBL4 clones), incubating the plate at 42°C for 7 hours, when the T-top agar overlay has set; then overlaying the plaques on the plate with a 5ml volume of SM buffer and finally incubating the plate + buffer at 4°C overnight. The addition of DMSO (to a final concentration of 7%) to the SM buffer overlay, recovered from the plate, allows the amplified phage stock to be stored at -70°C.
6.12 The Production of Recombinant gtII Lysogens and Crude Protein Lysates

Crude *E. coli* lysate, containing the recombinant antigenic protein molecule expressed by a gtII clone can be prepared by initially expressing the particular recombinant gtII clone as a lysogen in *E. coli* strain Y1089, i.e. the recombinant gtII DNA is incorporated into the genome of the *E. coli* host. The host strain, Y1089 was specifically constructed (Young and Davis, 1983a;b) to allow the production of lysogens and contains: a mutation which enhances the frequency of phage lysogeny (hflA150); the lacI gene product (the lac repressor) which prevents lac-Z directed gene expression (i.e. prevents expression of the beta-galactosidase gene and production of beta-galactosidase fusion proteins), until derepressed by the addition of IPTG to the cell culture and a deficiency in the lon protease which increases the stability of expressed recombinant fusion proteins.

A 10ml L-Broth culture (supplemented with 0.2% maltose) of *E. coli* Y1089 cells was prepared and incubated at 30°C overnight, resulting in the production of a culture containing c.10⁸ cells/ml. Aliquots of this culture (c.10⁴ cells) were then mixed with phage from an amplified, plaque-purified stock, at a multiplicity of 5 phage/cell as follows:

100μl *E. coli* Y1089 cells (c.10⁴) + 880μl L-Broth (+0.2% maltose) + 10mM MgCl₂ + 5 x 10⁴ phage.
This infection mixture was then incubated at 30°C for 20 minutes, to allow adsorption of the phage to the cells to occur and then aliquots were plated out onto L-Agar plates (at a density of c.200 cells per plate) which were then incubated at 30°C overnight.

Following incubation at 30°C overnight, colonies grew on each of the plates and these were subsequently tested to allow the identification of lysogenic colonies. With the aid of toothpicks, those colonies which grew at 30°C were stabbed onto two sets of L-Agar plates; one set of plates was incubated at 30°C overnight and the second set of plates was incubated at 42°C overnight. The gtII vector produces a temperature-sensitive phage repressor (cl857) which is functional at 30°C but inactive at 42°C, consequently lysogenic colonies are those capable of growth at 30°C but not able to grow at 42°C.

To prepare crude protein lysates from the lysogenic clones, a 2ml L-Broth culture of each lysogen was prepared and grown at 30°C overnight. Aliquots (300μl) of these overnight cultures were used to inoculate 30ml volumes of L-Broth, which were then incubated at 30°C for 3 hours, i.e. until the optical density of the cultures was 0.5 at 600nm. The cultures were incubated at 42°C for 20 minutes, to inactivate the temperature-sensitive phage repressor (cl857) and to induce the lysogens. IPTG was then added to the cultures, to a final concentration of 10mM, to derepress the lac repressor and thus stimulate lac-Z directed gene expression and the production of the recombinant antigens.
Following the addition of IPTG, the cultures were incubated at 37°C for 30 minutes, to allow large quantities of the expressed phage products to be accumulated within the lysogenic cells. Unfortunately, despite the gtII vector containing a mutation (S100) which renders E.coli Y1089 cells lysis defective, the Y1089 lysogens do undergo lysis if incubation at 37°C is prolonged (a consequence of the 'leakiness' of the S100 mutation). Thus, following incubation of the cultures at 37°C for 30 minutes and prior to the onset of lysis, the cells were harvested by centrifugation at 8000 rpm for 5 minutes (at 37°C) and the pellets were then resuspended in 1ml volumes of 50mM Tris-HClpH 8, to concentrate the cell samples. The cell suspensions were then incubated in liquid nitrogen for c.1 hour and were then thawed, to cause complete lysis of the induced lysogens and the release of the expressed recombinant antigens. These concentrated crude protein lysate samples were then incubated at -70°C until required.
6.13 Detection of Proteins on SDS-Polyacrylamide Gels with Antibodies

This procedure involves three distinct steps: (1) the separation of proteins in a sample, by electrophoresis through an SDS-polyacrylamide (SDS-PAGE) gel (Laemmli, 1970); (2) the electrophoretic transfer of the separated proteins, from the SDS-PAGE gel to a nitrocellulose filter, by Western blotting (Towbin et al., 1979) and (3) detection of specific antigens, following the incubation of the Western blot in the appropriate primary and secondary antibody solutions and the subsequent development of the Western blot in the appropriate colour development solution.

6.13.1 SDS-Polyacrylamide Gel Electrophoresis

Reagents:

Acrylamide solution: 30% acrylamide; 0.8% bis-acrylamide

4X stacking gel buffer: 60.5g/litre Tris base, adjusted to pH 6.7;

0.4% SDS

4X separating gel buffer: 181.6g/litre Tris base, adjusted to pH 8.8; 0.4% SDS

TEEXED

10% Ammonium persulphate

5X SDS-PAGE running buffer: 60.5g/litre Tris base; 37.5g/litre glycine; 5g/litre SDS

PBS: 8g/litre NaCl; 0.2g/litre KCl; 0.2g/litre KH₂PO₄; 1.15g/litre Na₂HP₂O₄ pH 7.3
1X SDS PAGE loading buffer: 75 mM Tris-HCl pH 6.8; 5% 2-mercaptoethanol; 2% SDS; 10% glycerol; 0.002% bromophenol blue

Procedure

SDS-PAGE gels were prepared using a BRL Vertical Gel Kit (model V16-2). The gels consisted of two parts: a lower separating gel and an upper stacking gel. The percentage of acrylamide used in the separating gel was determined by the size of the protein molecules to be separated: (1) a 3-5% separating gel optimises the separation of protein molecules large than 100KD; (2) a 5-12% separating gel optimises the separation of protein molecules of 30KD-150KD; (3) a 10-15% separating gel will optimise the separation of protein molecules of 10KD-80KD and (4) a 15% separating gel (or larger) will optimise the separation of proteins smaller than 15KD. Table 6 below, describes the production of the separating and stacking gels, commonly used in this project.
Table 6: Recipes for SDS-PAGE Gels

<table>
<thead>
<tr>
<th></th>
<th>5% Stacking Gel</th>
<th>Percentages of Separating Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>5.0ml</td>
<td>6.7ml</td>
</tr>
<tr>
<td>4X Stacking Gel Buffer</td>
<td>7.5ml</td>
<td>10.0ml</td>
</tr>
<tr>
<td>4X Separating Gel Buffer</td>
<td></td>
<td>10.0ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>17.0ml</td>
<td>22.8ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>30μl</td>
<td>40μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate</td>
<td>300μl</td>
<td>400μl</td>
</tr>
<tr>
<td>TOTAL VOLUME</td>
<td>30ml</td>
<td>40ml</td>
</tr>
</tbody>
</table>
The two 19.5 x 16cm glass plates were washed in distilled water and ethanol and spacers (1.5mm thick) were inserted between them and held in place by bulldog clips, to form the sides and bottom edge of the gel. A 40ml volume of the appropriate percentage separating gel was prepared and immediately poured between the glass plates, to within 4cm of the top of the plates. The acrylamide solution was then overlayed with c.300µl of water-saturated butan-1-ol, to form a sharp edge to the separating gel. When the gel had set (c.10-15 minutes) the butan-1-ol layer was thrown off and then the 5% stacking gel mix was prepared and poured. A 20 well comb was inserted immediately and the gel was allowed to set for c.10-15 minutes. When the gel had set the comb, bottom spacer and bulldog clips were removed and the glass plates were inserted into the BRL Vertical Gel Tank apparatus and held in place by bulldog clips. Finally, 1X SDS-PAGE running buffer was added to both the upper and lower reservoirs.

Samples of recombinant plasmid clones (e.g. pBR322, pUC13 etc) were initially grown as 2ml L-Broth overnight cultures and then 1ml aliquots of these overnight cultures were pelleted (in an Eppendorf centrifuge) and the pellets were resuspended in 500µl volumes of PBS. These samples were then repelleted and the pellets resuspended in 100µl volumes of 1X SDS-PAGE loading buffer. On the other hand, 50µl aliquots of the crude protein lysate samples of recombinant gtII clones were simply mixed with an equal volume of 2X SDS-PAGE loading buffer. Prior to loading 50µl aliquots of each sample per well, the samples were boiled for 5 minutes. An aliquot of a standard protein marker mixture (Table 7) was always loaded also.
Electrophoresis was carried out at 80V for 2 hours through the stacking gel and then at 160V for c.2 hours through the separating gel, i.e. until the tracking dye was within 1 cm from the bottom edge of the gel.

Table 7: Standard Protein Marker Mixtures

<table>
<thead>
<tr>
<th>Marker Mixture</th>
<th>Proteins</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Molecular Weight</td>
<td>Myosin (rabbit muscle)</td>
<td>205000</td>
</tr>
<tr>
<td>Standard Protein Mixture</td>
<td>beta-galactosidase (E.coli)</td>
<td>116000</td>
</tr>
<tr>
<td>Sigma</td>
<td>phosphorylase b (rabbit muscle)</td>
<td>97400</td>
</tr>
<tr>
<td></td>
<td>albumin (bovine)</td>
<td>66000</td>
</tr>
<tr>
<td></td>
<td>albumin (egg)</td>
<td>45000</td>
</tr>
<tr>
<td></td>
<td>carbonic anhydrase (bovine erythrocytes)</td>
<td>29000</td>
</tr>
<tr>
<td>Low Molecular Weight</td>
<td>albumin (bovine)</td>
<td>66000</td>
</tr>
<tr>
<td>Standard Protein Mixture</td>
<td>albumin (egg)</td>
<td>45000</td>
</tr>
<tr>
<td>Sigma</td>
<td>glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)</td>
<td>36000</td>
</tr>
</tbody>
</table>
|                             | carbonic anhydrase (bovine erythrocytes) | 29000 
|                             | trypsinogen (bovine pancreas)           | 24000            |
|                             | trypsin inhibitor (soybean)             | 20100            |
|                             | alpha-lactalbumin (bovine milk)         | 14200            |
Western Blotting SDS-Polyacrylamide Gels

Reagents:

1X Electroblotting buffer: 3.03g/litre Tris base; 14.4g/litre glycine; 20% (v/v) methanol

Procedure

Following electrophoresis, the SDS-PAGE gel was removed from the electrophoresis tank and incubated in a 500ml volume of 1X electroblotting buffer, for 30 minutes. Two sheets of Whatman No. 1 filter paper and a nitrocellulose filter were cut to the dimensions of the SDS-PAGE gel (15 x 17cm) and were similarly incubated in electroblotting buffer. Next, the gel and filter papers were placed within an electroblot gel holder (Bio-Rad Trans-Blot Cell) in the following order: an electroblotting buffer-saturated fiber pad; a sheet of buffer-saturated filter paper; the buffer-saturated SDS-PAGE gel; buffer-saturated nitrocellulose filter; a second buffer-saturated filter paper and then finally, a second buffer-saturated fiber pad. The electroblot gel holder was then closed and placed within the electroblotting tank (with the SDS-PAGE gel facing the cathode) containing c.3 litres of 1X electroblotting buffer. Blotting was then carried out at 30V overnight at 4°C.
6.13.3 Screening Western Blots with Antibodies

Reagents:

TBS : 50mM Tris-HCl pH 7.5; 150mM NaCl
TBST : 50mM Tris-HCl pH 7.5; 150mM NaCl; 0.05% Tween 20
BSA : Bovine serum albumin
Primary antibodies : polyclonal or monoclonal antibodies
Secondary antibodies : biotin conjugated - , alkaline phosphatase conjugated - or horseradish peroxidase conjugated secondary antibodies
Colour development solution (horseradish peroxidase or alkaline phosphatase)

Procedure

Following electrophoresis the nitrocellulose filter was removed from the blotting apparatus and incubated for 60 minutes in a solution of TBST containing 3% BSA, to saturate the non-specific protein binding sites on the filter. The filter was then removed from the blocking solution and incubated for 60 minutes in a solution of TBST + 3% BSA containing an appropriate dilution of the primary antibodies to be used. Next, the filter was washed twice with TBST (each wash for 5 minutes), then incubated in TBST containing the appropriate secondary antibodies. Following incubation in TBS for 60 minutes (to remove unbound secondary antibodies), the filter was incubated in the appropriate colour
development solution and antigenic proteins were detected as coloured bands (blue/purple) on the filter.

The position of the protein marker bands can be located by staining the nitrocellulose filter with amido black: 0.1% amido black dye; 45% methanol; 10% acetic acid (Schaffner and Weissmann, 1973). Following staining for 2-3 minutes, the filter is rinsed in distilled water for 1 minute; washed three times (each for only 1 minute), in the amido black destain solution (90% methanol; 2% acetic acid) and then rinsed in distilled water. Thus, amido black staining of the nitrocellulose filter results in the determination of the size of the antigenic proteins.
6.14 Protein Staining SDS-Polyacrylamide Gels

Reagents:

PAGE Blue 83 stain: 0.2% PAGE Blue 83 (BDH); 95% ethanol
Destain solution: 45% methanol; 10% acetic acid

Procedure

If Western blotting is not to be carried out, then SDS-polyacrylamide (SDS-PAGE) gels, electrophoresed as described in section 6.13.1, can be stained with PAGE Blue 83 dye in order to allow the separated protein bands to be visualised. Immediately prior to use, the PAGE Blue 83 stain is mixed with an equal volume of 20% acetic acid and the SDS-PAGE gel is then incubated in this solution for c.3 hours. The stained gel is then incubated in the destain solution until no further dye is removed from the gel and the protein bands can be visualised.
6.15 Preparation of a Standard Protein Curve

Reagents:

- BSA Solution: 5mg/ml bovine serum albumin in distilled water
- Bio-Rad Protein Assay Kit

Procedure

The protein assay dye solution was prepared by diluting one volume of the Bio-Rad Dye Reagent concentrate with four volumes of distilled water and then filtering this solution through a Whatman No. 1 filter paper. 5ml volumes of this diluted, filtered dye solution were then aliquoted into test tubes. Using the 5mg/ml BSA solution, a range of protein concentrations were prepared: 1mg/ml; 800μg/ml; 600μg/ml; 400μg/ml and 200μg/ml BSA solutions and 0.1ml volumes of these solutions were aliquoted into those test tubes containing the dye solution, which were then mixed by inversion.

Using disposable plastic cuvettes the absorbance of each sample was read at 595nm and the readings obtained for 0.1ml aliquots of the five protein samples described above, are listed in Table 8 below. Furthermore, using these OD595 values a standard protein curve was drawn: OD595 values versus protein concentration (Figure 4).

The concentrations of other protein solutions were subsequently determined by mixing 0.1ml aliquots of the respective protein solutions
with 5ml volumes of the diluted, filtered dye solution as described above; determining the absorbances of these samples at 595nm and by then reading the protein concentrations, corresponding to the OD595 values, from the standard protein curve.

Table 8: OD595 Readings for Known BSA Concentrations

<table>
<thead>
<tr>
<th>Sample</th>
<th>μg's Protein</th>
<th>OD595</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1ml of a 200μg/ml BSA solution</td>
<td>20</td>
<td>0.274</td>
</tr>
<tr>
<td>0.1ml of a 400μg/ml BSA solution</td>
<td>40</td>
<td>0.512</td>
</tr>
<tr>
<td>0.1ml of a 600μg/ml BSA solution</td>
<td>60</td>
<td>0.705</td>
</tr>
<tr>
<td>0.1ml of a 800μg/ml BSA solution</td>
<td>80</td>
<td>0.896</td>
</tr>
<tr>
<td>0.1ml of a 1mg/ml BSA solution</td>
<td>100</td>
<td>1.070</td>
</tr>
</tbody>
</table>
Figure 4: Standard Protein (BSA) Curve

OD 595

0 0.2 0.4 0.6 0.8 1

0 20 40 60 80 100

μg BSA
6.16 Determining the Activity of Chloramphenicol Acetyltransferase Expressed by Recombinant Clones

6.16.1 Preparation of Crude Protein Lysates (Shaw, 1975)

Reagents:

- L-Broth
- 20% Glucose
- TDTT buffer: 50mM Tris-HCl pH 7.8; 30μM DL-dithiothreitol

Procedure

Samples (recombinant pKK232-8 clones) to be assayed for the enzyme chloramphenicol acetyltransferase (CAT) were initially cultured at 37°C overnight in L-Broth supplemented with 0.2% glucose. Following overnight growth, 0.1ml aliquots of these cultures were used to inoculate 10ml volumes of L-Broth containing 0.2% glucose and these new cultures were subsequently incubated at 37°C for 3 hours. The cells were then harvested by centrifugation at 4000 rpm (MSE Chilspin) for 10 minutes and the pelleted cells were then resuspended in 3ml volumes of TDTT buffer.

A 1.5ml volume of each sample was then aliquoted into a 1.6ml microfuge tube and the cells were sonicated (MSE sonicator) on ice, with 2 x 15 second pulses (power = medium; amplitude = 3). Each of the sonicated samples was then centrifuged for 10 minutes at 4°C in an
Eppendorf centrifuge, to pellet the cell debris. The supernatants (crude protein lysates) were subsequently recovered and stored in microfuge tubes at -20°C, until the chloramphenicol acetyltransferase (CAT) enzyme assay could be performed.

6.16.2 The Chloramphenicol Acetyltransferase Assay (Shaw, 1975)

Reagents:

Crude protein lysates
5mM Acetyl-CoA : 20mg acetyl-CoA (lithium salt) in 5ml of distilled water
10μM DTNB : 40mg 5,5'-dithiobis- (2 nitrobenzoic acid) in 10ml 1M Tris-HCl pH 7.8
5mM chloramphenicol : 32mg chloramphenicol in 20ml of distilled water

Procedure

A 10 ml volume of the chloramphenicol acetyltransferase (CAT) assay buffer was prepared : 1.0ml 10μM DTNB; 0.2ml 5mM acetyl-CoA; 8.8ml distilled water and was stored on ice until required. A 0.556ml volume of the CAT assay buffer was aliquoted into a quartz micro-cuvette which was then placed into a temperature-controlled sample chamber of a Pye Unicam spectrophotometer (PU8820) which was linked to a BBC microcomputer and an Epsom FX-80 printer. The assay buffer sample was allowed to warm up to the reaction temperature (37°C) for 2 minutes,
before a 40μl aliquot of crude protein lysate was added to the assay buffer sample. The absorbance of the sample at 412nm was subsequently recorded. After recording the absorbance for 300 seconds, a 24μl volume of 5mM chloramphenicol was added to the micro-cuvette and the absorbance of the reaction mixture was recorded for a further 300 seconds. Using software supplied by the manufacturer, the computer calculated the rate of acetylation before and after the addition of chloramphenicol to the reaction mixture and the change in the rate of acetylation was subsequently determined. The protein concentrations of the crude protein lysates were determined in accordance with the protocol described in section 6.15.
6.17 DNA Sequencing

6.17.1 Production of Recombinant M13mp-9 Molecules

M13 cloning systems have been developed by a number of workers (Gronenborn and Messing, 1978; Messing and Vieira, 1982; Messing et al., 1981) for the generation of single-stranded DNA templates suitable for sequencing by the Sanger "dideoxy method" (Sanger et al., 1977). M13 is a single-stranded filamentous phage and its life cycle can be exploited for the production of single-stranded DNA templates (Schreir and Cortese, 1979).

On entering a suitable host cell (e.g. E.coli JM101), with the aid of its F pilus, the M13 phage molecule is stripped of its protein coat. Within the host cell the single-stranded phage DNA is converted to a double-stranded replicative form (RF), which undergoes DNA replication, resulting in the production of c.100 RF molecules. Single-stranded DNA molecules are then packaged into phage coat proteins and extruded from the host without cell lysis.

Foreign DNA can be ligated into a suitable restriction enzyme site within one of the many double stranded RF DNA cloning vectors which are commercially available (e.g. M13mp-9, a 7.2Kb cloning vector, containing unique restriction sites for SmaI, XmaI, AccI, HincII, EcoRI, BamHI, HaeIII, SalI, PstI and HindIII) and the recombinant molecules produced can then be introduced in competent E.coli JM101 cells. The resultant phage growth will lead to the production of recombinant
molecules in both double-stranded and single-stranded forms. The recombinant phage molecules can be harvested and single-stranded DNA can be recovered, suitable for Sanger "dideoxy sequencing".

A 1µg aliquot of M13mp-9 DNA was digested with 10 units of restriction enzyme EcoRI for c.1.5 hours at 37°C and then an aliquot was removed and electrophoresed through a 1% agarose gel, confirming the vector was completely digested. The remainder of the vector was then CIAP treated.

Using the method described in section 6.4.1, phage lambda DNA was extracted from an amplified phage stock of gtII/M_hovis BCG clone 532-9. A 10µg aliquot of the resultant recombinant DNA was then digested with 240 units of EcoRI at 37°C overnight. The digest was then electrophoresed through a 0.8% agarose and the 3.5Kb insert DNA fragment was recovered from the gel, as described in section 6.5.6.

The following ligation reaction was subsequently prepared and incubated at 12°C overnight:

30ng M13mp-9 DNA, EcoRI-digested, CIAP-treated + 150ng clone 532-9 insert DNA, EcoRI-digested + 1µl 10mM ATP + 1µl 10X Ligation buffer + 1 unit T4-DNA ligase.
6.17.2 Transfection of Competent *E. coli* JM101 cells with Recombinant M13mp-9 Molecules

A 2ml volume of L-Broth was inoculated with *E. coli* JM101 cells and the culture was incubated at 37°C overnight. This overnight culture was subsequently used to inoculate a 40ml volume of pre-warmed L-Broth, which was then incubated at 37°C for 3 hours. At the same time a 20ml volume of pre-warmed L-Broth was inoculated with a 50μl aliquot of the overnight culture and this culture was incubated at 37°C, to provide fresh *E. coli* JM101 cells for use at the plating stage.

Following 3 hours growth the 40ml culture of *E. coli* JM101 cells was centrifuged at 3000 rpm for 5 minutes. The cell pellet was then resuspended in 20ml of 50mM CaCl₂ (ice-cold) and this suspension was incubated on ice for 20 minutes. The cells were then pelleted as before, then resuspended in a 4ml volume of 50mM CaCl₂ (ice-cold) and stored on ice until required (at this stage the cells are competent).

Aliquots (0.2ml) of competent cells were transferred to 1.6ml microfuge tubes and stored on ice. A 10μl aliquot of ligated DNA was then added to each 0.2ml volume of competent cells and the mixtures were incubated on ice for 40 minutes. The samples were then heated at 42°C for 3 minutes, then reincubated on ice. Aliquots of 100mM IPTG (10μl), 2% X-gal (30μl) and fresh *E. coli* JM101 cells (100μl) were added to each of the heated samples and these mixtures were then added to 3ml volumes of molten T-top agar and poured immediately onto pre-warmed L-Agar.
plates. When the overlays had set, the plates were incubated at 37°C overnight.

M13 does not lyse its host cell but the growth of the host is retarded as a consequence of supporting phage growth. Subsequently, when plated out, those cells infected with M13 will appear as areas of slower growth (looking like turbid plaques) on lawns of uninfected cells. Moreover, when plated out onto L-Agar plates containing IPTG and X-gal, non-recombinant M13 phage will be visualised as blue plaques but recombinant phage appear as colourless or "white" plaques.

6.17.3 Preparation of Single-Stranded M13 DNA Template

A 10ml volume of L-Broth was inoculated with *E. coli* JM101 cells and the culture was incubated at 37°C overnight. A 50μl aliquot of this culture was then added to a 5ml volume of pre-warmed L-Broth and 1.5ml aliquots of the resultant culture were dispensed into sterile bijoux. Infected cells from recombinant (colourless) plaques were picked from the L-Agar plates (see above), with the aid of toothpicks and used to inoculate the 1.5ml volumes of *E. coli* JM101 cells. These samples were then incubated (with vigorous shaking) at 37°C for 6-8 hours. The cultures were then transferred to microfuge tubes and centrifuged for 5 minutes at room temperature. The supernatants were recovered and recentrifuged, to ensure that all cells were removed (supernatants can be stored at 4°C at this stage). A 250μl aliquot of 20% polyethylene glycol 6000; 2.5M NaCl was subsequently added to each 1ml volume of supernatant and these mixtures were incubated on ice for 15 minutes.
The mixtures were then centrifuged for 10 minutes at 4°C (to pellet the phage particles) and the supernatants were discarded. The pellets were then resuspended in 100μl of TE buffer and a 100μl aliquot of a phenol/chloroform solution (100g Analar phenol + 100ml chloroform, equilibrated with TE buffer) was subsequently added. The samples were then mixed by vortexing (for 15 seconds), left at room temperature for 10 minutes, then revortexed and finally centrifuged for 5 minutes at room temperature, to separate the two layers. The aqueous (upper) layers were recovered and transferred to microfuge tubes containing 10μl of 3M sodium acetate (pH 6.0). A 250μl volume of ethanol was then added to the tubes, which were then incubated on ice for 30 minutes, to precipitate the phage DNA. The DNA was pelleted by centrifuging the ethanol precipitations at 4°C for 20 minutes, the supernatants were then discarded and the pellets resuspended in 100μl volumes of TE buffer. A 1μl aliquot of Proteinase K (5mg/ml stock) was added to each DNA sample, which were then incubated at room temperature for 30 minutes and the DNA samples were recovered following (i) phenol/chloroform extraction and (ii) ethanol precipitation (as described above).

The DNA pellets were resuspended in 25μl volumes of TE buffer and to check the quality and quantity of the prepared DNA, 2μl aliquots of each sample were analysed on 1% agarose gels.
**6.17.4 Sequencing Reactions**

**Materials**

(i) Stock dNTP and ddNTP nucleotides (purchased from BCL) were made up in distilled water (i.e. 10mM d/ddNTP stock solutions) and stored at -20°C.

(ii) Working solutions were prepared with distilled water (as described below) and stored at -20°C.

a) 0.5mM stocks of dTTP; dCTP; dGTP and dATP

b) dNTP* Mixes:

<table>
<thead>
<tr>
<th></th>
<th>T*</th>
<th>C*</th>
<th>G*</th>
<th>A*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5mM dTTP</td>
<td>1μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>+ 0.5mM dCTP</td>
<td>20μl</td>
<td>1μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>+ 0.5mM dGTP</td>
<td>20μl</td>
<td>20μl</td>
<td>1μl</td>
<td>20μl</td>
</tr>
<tr>
<td>+ TE buffer</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>

c) The Chase Mix consists of a mixture of the four dNTPs, each at a concentration of 0.5mM.

d) ddNTP working stocks are prepared according to requirement, e.g. for running a short gel (c.120 minutes), the following concentrations of the ddNTP nucleotides were used:
i.e. ddTTP 0.5mM
ddGTP 0.1mM
ddGTP 0.3mM
and ddATP 0.1mM

e) d/ddNTP Mixes: to each volume of dNTP* mix, add an equal volume of the corresponding ddNTP working stock solution.
e.g. 50μl T* + 50μl ddTTP
50μl G* + 50μl ddGTP
50μl C* + 50μl ddCTP
50μl A* + 50μl ddATP.

Procedure

For each DNA sample to be sequenced, a microfuge tube containing:
2μl M13 primer (17 nucleotide primer, from Celltech), 1μl 10X annealing buffer (100mM Tris-HCl pH 8.5; 50mM MgCl₂) and 7μl of the single-stranded M13 recombinant DNA template (section 6.17.3), was prepared and each tube was then incubated at 65°C for 90 minutes.

Following incubation, the annealed samples were removed from the 65°C water-bath (centrifuged briefly to bring any condensation to the bottom of the tube), cooled and then 0.5μl Klenow (c.3 units) and 10μCi 35S-dATP (Amersham, 600Ci/mmol) were added to each tube. A set of four microfuge tubes (one set for each annealed sample) were labelled, using a colour code: T = green; C = blue; G = black; A = red, and 2μl aliquots of the d/ddNTP mixes were dispensed into the appropriate tubes (i.e. 2μl d/ddTTP mix was dispensed into the T tube, ... etc.)
Equal volumes of the annealed samples were then dispensed into each of the four labelled tubes (containing the appropriate d/ddNTP mixes) and the resultant mixtures were incubated at room temperature for 20 minutes. Following incubation, a 2 µl aliquot of the Chase Mix was added to all of the tubes (i.e. T, C, G and A tubes) which were then incubated at room temperature for a further 20 minutes. To each tube, a 4µl aliquot of formamide dye mix (0.03% xylene cyanol; 0.03% bromophenol blue; 20mM EDTA in deionised formamide) was added and the tubes were then centrifuged briefly to mix the samples. Each sample was then placed in a boiling water bath for 4 minutes, prior to loading 4µl aliquots on a sequencing gel (see below.)

6.17.5 Preparation of the Sequencing Gel and Running Samples

The two, 40 x 20cm glass plates were scrubbed with detergent and hot water, rinsed with distilled water and then allowed to dry. One face of the notched plate was then wiped with Repelcote (carried out in the fume cupboard) and one face of the unnotched plate was wiped with a solution comprising : 10ml of ethanol; 150µl of 10% acetic acid; 30µl of gamma-methacryloxypropyl-trimethoxy silane (in the fume cupboard). Both plates were then wiped with ethanol and (when dry) assembled together (the treatment faces innermost), separated with plastic side spacers (1mm thick). The sides and bottom of the assembled plates were finally sealed with masking tape (bulldog clips were also used to hold the plates together).
A solution consisting of an 11.25ml volume of 40% acrylamide; 2% bis-acrylamide, a 7.5ml volume of 10X borate buffer and 31.5g of urea was prepared (and the final volume was made up to 75ml with distilled water), then gently swirled at 37°C to dissolve the urea. To this solution were added, a 420µl aliquot of a 10% ammonium persulphate solution (freshly prepared) and a 75µl aliquot of TEMED. The solution was mixed, then immediately poured between the assembled glass plates. A plastic comb was inserted between the plates to form wells (no deeper than 5mm) and the gel was left to polymerise for at least 45 minutes. When the gel had set, the masking tape was removed from the bottom of the glass plates, the plates were then clamped to the gel apparatus (Raven scientific), 1X borate buffer was poured into the top and bottom reservoirs and the comb was then carefully removed.

Following boiling for 4 minutes, 4µl aliquots of the T-, C-, G- and A- samples (for each of the annealed templates) were loaded into wells of the gel. The power was then switched on and the power pack set to 50V constant power, with the voltage set at 1800V and the current set at 40mA. The gel was then run until the lower marker dye (xylene cyanol) had run to within 1-2cm of the gel bottom (short gel = 120 minutes) or until the upper marker dye had run to within 1-2 cm of the gel bottom (medium gel = c.240 minutes).

After electrophoresis, the glass plates were removed from the gel apparatus and the plates were then dismantled. The gel remained stuck to the unnotched plate (as expected), and the plate + gel were then incubated in a fixing solution (10% methanol; 10% acetic acid) for
20 minutes. The gel was then rinsed thoroughly (under gently running, cold water) for 5 minutes, then dried in an oven at 80°C for 1 hour. The dried gel was then allowed to cool, prior to exposure to an X-ray film. The glass plate + dried gel was then placed in an X-ray cassette and overlaid with Fuji-RX film (film exposure was carried out in the dark). The length of exposure necessary was estimated by passing a radioactivity mini monitor over the dried gel, with counts in the range 10-100 c.p.s. (at the top of the gel) suggesting an exposure time of c.48 hours.
EXPERIMENTS

Section 7 Results Following the Comparison of Four Methods for the Extraction of M.bovis BCG DNA from Liquid Cultures of Cells

Four 250ml volumes of Sauton medium (section 5.3) were prepared and each was inoculated with a loopful of M.bovis BCG cells (from a Lowenstein-Jensen slope), then each culture was incubated at 37°C.

Following growth at 37°C for 3 weeks, each of the cultures was harvested separately, by centrifugation at 10000 rpm for 15 minutes. The supernatants were then discarded and the wet weight of M.bovis BCG cells was determined for each of the four samples. The values obtained are contained in Table 9.

Four methods were employed to extract M.bovis BCG DNA from the four pelleted cell samples and each method was slightly adapted from the previous one (section 6.2.1): Method I was based upon the use of an alkaline SDS solution to achieve lysis of the M.bovis BCG cells; Method II involved the addition of glycine to the M.bovis cell culture, 12 hours prior to harvesting the cells, to increase the susceptibility of the cells to lysis when treated with the alkaline SDS solution (Rastogi et al., 1983); Method III involved the use of both glycine and alkaline SDS, together with a lysosome solution, which further aided lysis of the cells and Method IV employed glycine, the lysosome solution and 2X Kirby mixture (a detergent solution which replaced alkaline SDS).
The four *M. bovis* BCG DNA pellets were subsequently resuspended in 0.5ml aliquots of TE buffer.

The purity of the four *M. bovis* BCG DNA samples was assessed spectrophotometrically by determination of the ratio of absorbance at 260nm to 280nm (i.e. A260/A280) for each sample. The A260 and A280 values are listed in Table 10 for 50μl aliquots of DNA preparations 1, 2 and 3 and for a 25μl aliquot of DNA preparation 4. The estimated DNA concentration (μg/ml) of each of the four DNA preparations is also listed in Table 10. Table 11 compares the amount of *M. bovis* BCG DNA obtained with each of the four extraction methods and reveals Method IV to be the most efficient. Consequently, this method was used whenever further aliquots of *M. bovis* BCG DNA were required.

The A260/A280 ratio of each DNA sample was calculated to be greater than 1.7 (Table 10), indicating that the four DNA samples were highly pure (i.e. free from protein contamination). Their suitability for use in cloning experiments was confirmed by the ability to digest aliquots (10μl) of each sample with restriction enzyme BamHI. Photograph 1 below, shows the *M. bovis* DNA, BamHI restriction fragments of each of the four digests, following their electrophoresis through a 1% agarose gel (section 6.5.5).
Table 9: Wet Weight of *M. bovis* BCG cells Harvested from Four Cultures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wet Weight <em>M. bovis</em> BCG cells (g) per 250ml Culture Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.36</td>
</tr>
<tr>
<td>2</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>0.28</td>
</tr>
<tr>
<td>4</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 10: A260 and A280 Readings of the Four *M. bovis* BCG DNA Samples

<table>
<thead>
<tr>
<th>DNA Sample</th>
<th>Dilution Factor</th>
<th>A260</th>
<th>A280</th>
<th>A260/A280 Ratio</th>
<th>DNA Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50µl DNA (Method I)</td>
<td>41</td>
<td>0.015</td>
<td>0.008</td>
<td>1.88</td>
<td>30.8</td>
</tr>
<tr>
<td>50µl DNA (Method II)</td>
<td>41</td>
<td>0.026</td>
<td>0.014</td>
<td>1.86</td>
<td>53.3</td>
</tr>
<tr>
<td>50µl DNA (Method III)</td>
<td>41</td>
<td>0.051</td>
<td>0.026</td>
<td>1.96</td>
<td>104.6</td>
</tr>
<tr>
<td>25µl DNA (Method IV)</td>
<td>81</td>
<td>0.029</td>
<td>0.016</td>
<td>1.81</td>
<td>117.5</td>
</tr>
</tbody>
</table>
Table 11: Amount of *M. bovis* BCG DNA Extracted from Four Aliquots of *M. bovis* BCG Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction Method</th>
<th>μg <em>M. bovis</em> BCG DNA per 250ml culture volume</th>
<th>μg <em>M. bovis</em> BCG DNA per gram wet weight cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Method I</td>
<td>15.4</td>
<td>42.8</td>
</tr>
<tr>
<td>2</td>
<td>Method II</td>
<td>26.6</td>
<td>95.0</td>
</tr>
<tr>
<td>3</td>
<td>Method III</td>
<td>52.3</td>
<td>186.8</td>
</tr>
<tr>
<td>4</td>
<td>Method IV</td>
<td>58.8</td>
<td>245.0</td>
</tr>
</tbody>
</table>
Photograph 1: Bam HI Restriction Digests of Four *M. bovis* BCG DNA Samples

**Track Number**

1 = Bam HI digest of *M. bovis* BCG DNA sample 1.
2 = Bam HI digest of *M. bovis* BCG DNA sample 2.
3 = Bam HI digest of *M. bovis* BCG DNA sample 3.
4 = Bam HI digest of *M. bovis* BCG DNA sample 4.
5 = Hind III digest of lambda DNA.
Section 8: Cloning M. bovis BCG DNA into Escherichia coli

Following the doubts raised by Clark-Curtiss et al. (1985) as to the ability of the E. coli transcription and translation apparatus to recognise mycobacterial transcription and translation signals, work was undertaken to demonstrate the usefulness of E. coli as a bacterial host for cloning and expressing mycobacterial genes.

Expression of a cloned, foreign gene (e.g. a mycobacterial gene) in a bacterial cell (e.g. E. coli) is controlled by the transcription and translation machinery of the host cell. Transcription requires the presence of a promoter site within the cloned DNA fragment, that can be recognised by the host's RNA polymerase. If such a site exists, then the host's RNA polymerase binds at this site, a local unwinding of the DNA occurs and transcription of the cloned DNA into a sequence of messenger RNA (mRNA) is initiated. Translation of the mRNA sequence, into a corresponding sequence of amino acids, is dependent upon the presence of (i) a ribosome-binding site and (ii) a translation start codon, within the mRNA sequence. If however either of these two processes (transcription or translation) is unable to function with a cloned foreign gene, then expression of that gene will not occur.

The ability of M. bovis BCG DNA fragments to act as promoters in E. coli was tested using the promoter probe vector pKK232-8 (Brosius, 1984). This vector, a pBR322-derived plasmid vector, contains an ampicillin-resistance gene (for selection); a promoterless chloramphenicol-resistance gene, which codes for chloramphenicol
acetyltransferase (CAT); a multiple cloning site (MCS), positioned 5'-proximal to the chloramphenicol-resistance gene; three translation stop codons, situated between the multiple cloning site and the start codon of the chloramphenicol-resistance gene, to prevent translational readthrough into the gene; transcription terminators and a ribosome-binding site (Figure 5).

When a DNA fragment, containing a functional promoter sequence, is ligated into one of the cloning sites upstream from the promoterless chloramphenicol-resistance gene, cells carrying such a recombinant plasmid will acquire resistance to chloramphenicol. Consequently, BamHI restriction fragments of \textit{M. bovis} BCG DNA were prepared then ligated into the BamHI site within the multiple cloning site and \textit{E. coli} cells were transformed with the resultant recombinant molecules. Transformants harbouring \textit{M. bovis} BCG promoter sequences were subsequently identified by their ability to grow on media containing chloramphenicol.
Figure 5: Simplified Restriction Map of the Plasmid pKK232-8
(from Kieser et al., 1986)

Notes: amp = ampicillin-resistance gene; cat = promoterless chloramphenicol resistance gene, which codes for chloramphenicol acetyltransferase (CAT); the arrows indicate the direction in which the antibiotic resistance genes are transcribed.
8.1 The Cloning of M. bovis BCG DNA into the Promoter-Probe Plasmid pKK232-8

To determine whether mycobacterial promoter sequences were recognised by E. coli RNA polymerase, M. bovis BCG DNA was partially digested with restriction enzyme BamHI (section 6.5.3) and then an aliquot of the resultant restriction fragments was ligated into BamHI-digested, calf intestinal alkaline phosphatase (CIAP) treated pKK232-8, in accordance with the procedure described in section 6.5.4. To provide a comparison for the M. bovis BCG cloning experiment, BamHI restriction fragments of E. coli HB101 DNA were similarly prepared and ligated into pKK232-8 (Table 12).

Following overnight incubation at 12°C, the two ligation mixtures (BCG and HB101) and the four controls were used to transform E. coli HB101 cells. Competent cells were produced and transformation mixtures were prepared (see below) then subjected to the Kushner transformation procedure, as described in section 6.6.2.

**Transformation Mixtures:**

A = Ligation mixture : BCG + 200µl competent cells (E. coli HB101)

B = Ligation mixture : HB101 + 200µl competent cells

C = Control No. 1 + 200µl competent cells

D = Control No. 2 + 200µl competent cells

E = Control No. 3 + 200µl competent cells

F = Control No. 4 + 200µl competent cells
Following transformation, the mixtures were centrifuged and the cell pellets were resuspended in 0.1ml volumes of L-Broth, which were then plated out onto L-Agar plates containing 50μg/ml ampicillin. The plates were then incubated at 37°C overnight.

Table 13 below, lists the total number of ampicillin-resistant transformants obtained from (1) the BCG and HB101 cloning experiments and from (2) the four control experiments. Each of the control experiments gave extremely good results: (i) the results of Control No. 1 confirmed that competent E.coli HB101 cells had been prepared and that the transformation procedure was working; (ii) the results of Controls No. 2 and No. 3 revealed that the T4-DNA ligase was working and (iii) the results of Control No. 4 indicated that the preparation of the plasmid had been extremely good. From the results of the four controls, it was anticipated that a high proportion of the 1043 M.bovis BCG, ampicillin-transformants would carry inserted foreign DNA, within their pKK232-8 plasmids.
Table 12: Ligation Mixtures and Controls: Ligating *M. bovis* BCG DNA and *E. coli* HB101 DNA into the Plasmid pKK232-8

<table>
<thead>
<tr>
<th>Ligation Mixtures (µl)</th>
<th>Controls (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BCG</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>pKK232-8, BamHI-digested, CIAP-treated</td>
<td>5 (50ng)</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG DNA, BamHI-digested</td>
<td>1 (100ng)</td>
</tr>
<tr>
<td><em>E. coli</em> HB101 DNA, BamHI-digested</td>
<td></td>
</tr>
<tr>
<td>pKK232-8, uncut</td>
<td></td>
</tr>
<tr>
<td>pKK232-8, BamHI-digested</td>
<td></td>
</tr>
<tr>
<td>5mM ATP</td>
<td>1</td>
</tr>
<tr>
<td>10X Ligation buffer</td>
<td>1</td>
</tr>
<tr>
<td>T4-DNA Ligase</td>
<td>1</td>
</tr>
<tr>
<td>distilled water</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 13: The Number of Ampicillin-Resistant Colonies: Cloning

M. bovis BCG DNA and E. coli HB101 DNA into pKK232-8

<table>
<thead>
<tr>
<th>Cloning Experiments</th>
<th>Number of Ampicillin-Resistant Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCG:</strong></td>
<td></td>
</tr>
<tr>
<td>BamHI-digested, CIAP-treated</td>
<td></td>
</tr>
<tr>
<td>pKK232-8 + BamHI-digested</td>
<td></td>
</tr>
<tr>
<td>M. bovis BCG DNA</td>
<td>1043</td>
</tr>
<tr>
<td><strong>HB101:</strong></td>
<td></td>
</tr>
<tr>
<td>BamHI-digested, CIAP-treated</td>
<td></td>
</tr>
<tr>
<td>pKK232-8 + BamHI-digested</td>
<td></td>
</tr>
<tr>
<td>E. coli HB101 DNA</td>
<td>461</td>
</tr>
<tr>
<td><strong>Controls:</strong></td>
<td></td>
</tr>
<tr>
<td>1: pKK232-8 uncut</td>
<td>312</td>
</tr>
<tr>
<td>2: pKK232-8 cut, not ligated</td>
<td>4</td>
</tr>
<tr>
<td>3: pKK232-8 cut, ligated</td>
<td>115</td>
</tr>
<tr>
<td>4: pKK232-8 cut, CIAP-treated, ligated</td>
<td>0</td>
</tr>
</tbody>
</table>
8.2 The Selection of Ampicillin-Resistant Transformants with Cloned Promoter Activity

In order to identify transformants containing cloned DNA fragments with promoter activity, the 1043 pKK232-8/M. bovis BCG ampicillin-resistant transformants and the 461 pKK232-8/E. coli HB101 ampicillin-resistant transformants were rescreened to assess their resistance to chloramphenicol.

Each of the transformants was inoculated (with the aid of toothpicks), onto L-Agar plates containing 5µg/ml chloramphenicol and the plates were then incubated at 37°C overnight. Following overnight incubation, 170 pKK232-8/M. bovis BCG transformants were determined to be resistant and 873 pKK232-8/M. bovis BCG transformants were sensitive to 5µg/ml chloramphenicol. Similarly, 115 pKK232-8/E. coli HB101 transformants were resistant and 346 pKK232-8/E. coli HB101 transformants were sensitive to 5µg/ml chloramphenicol. Consequently, 170 pKK232-8/M. bovis BCG transformants had been identified with mycobacterial promoter activity, functional in E. coli.

To calculate the percentage of the 1043 M. bovis BCG transformants and the 461 E. coli HB101 transformants with cloned promoter activity, it was first necessary to determine the percentage of transformants with cloned insert DNA fragments. By employing the method described in section 6.3.2., plasmids were extracted from 19 pKK232-8/M. bovis BCG transformants (i.e. 11 chloramphenicol-resistant and 8 chloramphenicol-sensitive) and 21 pKK232-8/E. coli HB101 transformants
(i.e. 11 chloramphenicol-resistant and 10 chloramphenicol-sensitive). Each of the extracted plasmid DNA samples was then digested with BamHI and finally electrophoresed through a 1% agarose gel to confirm the presence or absence of insert DNA fragments.

All 11 of the pKK232-8/M. bovis BCG chloramphenicol-resistant transformants contained plasmids with inserts (the average insert size was 5Kb) and similarly, all 11 of the pKK232-8/E. coli HB101 chloramphenicol-resistant transformants also contained plasmids with inserts (the average insert size was 7.5Kb). However, only 75% of the pKK232-8/M. bovis BCG and 50% of the pKK232-8/E. coli HB101 chloramphenicol-sensitive transformants contained plasmids with inserts (data not shown).

To determine the percentage of pKK232-8/M. bovis BCG transformants with cloned promoter activity, the following calculations were performed: (i) 75% of a sample of chloramphenicol-sensitive transformants were found to contain inserts, thus the total number of chloramphenicol-sensitive transformants containing inserts was estimated as 873 x 0.75 = 655; (ii) the total number of transformants containing inserts was calculated by adding the total number of chloramphenicol-resistant transformants (all of which contain inserts) to the total number of chloramphenicol-sensitive transformants containing inserts, i.e. 170 + 655 = 825; (iii) the percentage of the 825 transformants (all containing inserts) that are chloramphenicol-resistant = \( \frac{170}{825} \times 100 = 21\% \).
Consequently, 21% of the total number of transformants with cloned \textit{M. bovis} BCG inserts, had cloned promoter activity, capable of conferring resistance to at least 5\textmu g/ml chloramphenicol. This result demonstrates that \textit{E. coli} RNA polymerase is able to recognise and utilise some mycobacterial transcription signals, confirming that \textit{E. coli} is a suitable host for the cloning and expression of mycobacterial genes.

Following a similar calculation to the one described above, 40% of the total number of pKK232-8/\textit{E. coli} HB101 transformants with cloned \textit{E. coli} HB101 inserts, were determined to have cloned promoter activity, capable of conferring resistance to at least 5\textmu g/ml chloramphenicol.

The work described above was published in the \textit{Journal of Bacteriology} : 168, 72-80, 1986, in a collaborative study with Tobias Kieser and David A. Hopwood. Our collaborators cloned \textit{M. bovis} BCG DNA restriction fragments into a Streptomyces promoter-probe plasmid and proceeded to transform \textit{S. lividans} cells with the resultant recombinant molecules. They also cloned \textit{S. lividans} DNA restriction fragments into the promoter-probe plasmid and transformed \textit{S. lividans} cells in an identical manner. Furthermore they calculated that 69% of their resultant \textit{M. bovis} BCG transformants contained \textit{M. bovis} BCG DNA inserts with cloned promoter activity, compared to 78% of their \textit{S. lividans} transformants containing \textit{S. lividans} DNA inserts with cloned promoter activity. From the similarity between the \textit{M. bovis} BCG transformants and \textit{S. lividans} transformants containing inserts with promoter activity, they concluded that the majority of \textit{M. bovis} BCG promoters are recognised and utilised by \textit{S. lividans} RNA polymerase and accordingly suggested that
S. lividans may prove to be a more suitable host than *E. coli* for cloning and expressing mycobacterial genes.

The relative strengths of the 170 cloned *M. bovis* BCG promoters and the 115 cloned *E. coli* HB101 promoters in *E. coli*, were assessed by testing each of the individual clones for resistance to increasing concentrations of chloramphenicol. With the aid of toothpicks, L-Agar plates containing different concentrations of chloramphenicol (5-1000μg/ml), were inoculated with each of the individual clones. The plates were then incubated at 37°C overnight. Table 14 lists the number of clones with promoter activity at each chloramphenicol concentration, furthermore these values were expressed as percentages of the total number of promoter active clones tested (i.e. 170 *M. bovis* BCG and 115 *E. coli* HB101 clones were tested). Although this table shows that the majority of the cloned promoters (both *M. bovis* BCG and *E. coli* HB101 promoters) were only weakly active, a few were shown to be highly active, in particular one cloned *M. bovis* BCG promoter (clone XIII/1) was discovered to be capable of conferring resistance to 600μg/ml chloramphenicol. Moreover, one of the cloned *E. coli* HB101 promoters was capable of conferring resistance to 800μg/ml chloramphenicol (i.e. clone 66/3/150).
### Table 14: Relative Strengths of the Cloned Promoters: Resistance to Increasing Concentrations of Chloramphenicol

<table>
<thead>
<tr>
<th>Chloramphenicol Concentration (µg/ml)</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
<th>500</th>
<th>600</th>
<th>800</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovis BCG clones resistant at each concentration</td>
<td>170</td>
<td>56</td>
<td>24</td>
<td>17</td>
<td>15</td>
<td>14</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% M. bovis BCG clones resistant at each concentration</td>
<td>100</td>
<td>32.9</td>
<td>14.1</td>
<td>10</td>
<td>8.8</td>
<td>8.2</td>
<td>5.3</td>
<td>3.5</td>
<td>2.3</td>
<td>1.2</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. coli HB101 clones resistant at each concentration</td>
<td>115</td>
<td>65</td>
<td>46</td>
<td>37</td>
<td>35</td>
<td>33</td>
<td>27</td>
<td>17</td>
<td>14</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>% E. coli HB101 clones resistant at each concentration</td>
<td>100</td>
<td>56.5</td>
<td>40</td>
<td>32.2</td>
<td>30.4</td>
<td>28.7</td>
<td>23.5</td>
<td>14.8</td>
<td>12.2</td>
<td>3.5</td>
<td>1.7</td>
<td>0.9</td>
<td>-</td>
</tr>
</tbody>
</table>
6.3 Measurement of the Specific Activity of the Chloramphenicol Acetyltransferase (CAT) Enzyme Expressed by Each of Twelve Clones

The enzyme chloramphenicol acetyltransferase (CAT) inactivates the antibiotic chloramphenicol by catalysing the formation of acetylated derivatives which are devoid of antibiotic activity (they cannot bind to bacterial ribosomes and therefore fail to inhibit polypeptide elongation). A number of clones (with cloned promoter activity) were subsequently assayed, according to the spectroscopic method described by Shaw (1975) (see section 6.16.2), in order to describe, if possible, a relationship between the number of units of CAT expressed by a clone and the subsequent level of chloramphenicol-resistance achieved by that same clone.

Spectrophotometrically the most convenient method for quantitating the rate of chloramphenicol acetylation, as catalysed by the CAT enzyme, involves measurement of the increase in absorbance at 412nm, as the yellow product 5-thio-2-nitrobenzoate (this compound has a molar extinction coefficient of 13600 at 412nm) is produced as a consequence of the following reactions:

1. Chloramphenicol + acetyl-S-CoA ---→ Chloramphenicol 3-acetate + HS-CoA
2. HS-CoA +

5,5'-dithiobisnitrobenzoic acid — mixed disulphide of CoA and thio-
(DNDB)
nitrobenzoic acid + 5-thio-2-
nitro-benzoate (yellow compound)

8.3.1 Production of Crude Protein Lysates and Determination of Total
Protein Concentrations

Six pKK232-8/M. bovis BCG clones and six pKK232-8/E. coli HB101
clones, each with cloned promoter activity, were selected on the basis
of their previously determined chloramphenicol-resistance levels:

pKK232-8/BCG clone XIII/1; resistant to 600µg/ml chloramphenicol
pKK232-8/BCG clone XIII/3; resistant to 500µg/ml chloramphenicol
pKK232-8/BCG clone XIII/2; resistant to 400µg/ml chloramphenicol
pKK232-8/BCG clone 87/4/160; resistant to 100µg/ml chloramphenicol
pKK232-8/BCG clone 33/4/160; resistant to 50µg/ml chloramphenicol
pKK232-8/BCG clone 52/14/90; resistant to 25µg/ml chloramphenicol

and

pKK232-8/HB101 clone 66/3/150; resistant to 800µg/ml chloramphenicol
pKK232-8/HB101 clone IX/1; resistant to 600µg/ml chloramphenicol
pKK232-8/HB101 clone 2/3/150; resistant to 500µg/ml chloramphenicol
pKK232-8/HB101 clone III/1; resistant to 400µg/ml chloramphenicol
pKK232-8/HB101 clone 105/6/150; resistant to 50µg/ml chloramphenicol
pKK232-8/HB101 clone 59/98/150; resistant to 25µg/ml chloramphenicol
A 10ml L-Broth culture of each of the above clones was prepared. Following 3 hours incubation at 37°C the cells were pelleted, resuspended in buffer and then sonicated, according to the method described in section 6.16.1. Following sonication the cell debris was pelleted and each of the crude protein lysates was recovered.

The absorbance at 595nm was determined for 0.1ml aliquots of each of the twelve crude protein lysates, according to the procedure described in section 6.15 and the values obtained are listed in Table 15. Using a BSA standard protein curve (prepared as described in section 6.15), each of the A595 values was then expressed as a protein concentration (mg total protein per ml crude lysate).
### Table 15: Total Protein Concentrations of the Crude Protein Lysates

<table>
<thead>
<tr>
<th>Sample</th>
<th>A595</th>
<th>mg total protein per ml crude lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK232-8/BCG clone XIII/1</td>
<td>0.464</td>
<td>0.37</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/3</td>
<td>0.838</td>
<td>0.75</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/2</td>
<td>0.831</td>
<td>0.74</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 87/4/160</td>
<td>0.682</td>
<td>0.59</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 33/4/160</td>
<td>0.802</td>
<td>0.71</td>
</tr>
<tr>
<td>pKK232-8/BCG clone '52/14/90</td>
<td>0.378</td>
<td>0.28</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 66/3/150</td>
<td>0.668</td>
<td>0.58</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone IX/1</td>
<td>0.647</td>
<td>0.56</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 2/3/150</td>
<td>0.695</td>
<td>0.60</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone III/1</td>
<td>0.781</td>
<td>0.69</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 105/6/150</td>
<td>0.900</td>
<td>0.81</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 59/98/150</td>
<td>0.853</td>
<td>0.76</td>
</tr>
</tbody>
</table>
8.3.2 Calculation of the Chloramphenicol Acetyltransferase Activity Expressed by the Twelve Clones

The CAT assay was performed as described in Section 6.16.2, using 40μl aliquots of each of the twelve crude lysate samples. Each crude lysate aliquot was added to a 1ml quartz cuvette containing a 0.556ml aliquot of the CAT assay reaction buffer and the absorbance at 412nm was recorded (for each of the samples) for 5 minutes, in order to determine the background rate of acetylation. A 24μl aliquot of chloramphenicol was then added to the quartz cuvette (the total reaction volume was now 620μl) and the absorbance of the assay mixture was recorded for a further 5 minutes. Figure 6 shows the CAT assay curve for pKK232-8/BCG clone XIII/3.

The rate of increase in absorption at 412nm, prior to the addition of the chloramphenicol was determined and this value was subtracted from the value for the rate of increase in absorption at 412nm, observed after the addition of the chloramphenicol, thus:

\[ \Delta \text{OD}_{412} = (\text{rate of increase in absorption after the addition of chloramphenicol}) - (\text{rate of increase in absorption before the addition of chloramphenicol}) \]

The observed net change in absorbance at 412nm (i.e. \( \Delta \text{OD}_{412} \)) was subsequently divided by the extinction coefficient value of the yellow product, 5-thio-2-nitrobenzoate, i.e. 0.0136, to express the \( \Delta \text{OD}_{412} \) value in nmoles per minute of chloramphenicol-dependent 5,5'-
dithionitrobenzoic acid reacted. This value is equivalent to the rate of chloramphenicol acetylation.

One unit of chloramphenicol acetyltransferase acetylates 1 µmole of chloramphenicol per minute (Shaw, 1975), consequently to determine the number of units of this enzyme, contained per ml of each crude lysate sample, each of the ΔOD412 values was subsequently adjusted to account for the volume of crude lysate used in each assay (40 µl).

i.e. (ΔOD412) x (1/0.0136) x (620/40) = nmoles/min/ml.

Table 16 contains the ΔOD412 values for each of the twelve CAT assays. Furthermore the CAT activities have been expressed in (i) nmoles/min/ml and (ii) enzyme units per ml crude lysate.
Table 16: CAT Assay Results: Units of Chloramphenicol Acetyltransferase per ml of Crude Lysate

<table>
<thead>
<tr>
<th>Sample</th>
<th>ΔOD412</th>
<th>CAT Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmoles/min/ml</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/1</td>
<td>0.031</td>
<td>35.6</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/3</td>
<td>0.047</td>
<td>53.4</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/2</td>
<td>0.038</td>
<td>43.1</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 87/4/160</td>
<td>0.008</td>
<td>9.2</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 33/4/160</td>
<td>0.006</td>
<td>7.4</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 52/14/90</td>
<td>0.008</td>
<td>0.9</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 66/3/150</td>
<td>0.087</td>
<td>99.2</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone IX/1</td>
<td>0.057</td>
<td>65.2</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 2/3/150</td>
<td>0.042</td>
<td>48.1</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone III/1</td>
<td>0.048</td>
<td>54.9</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 105/6/150</td>
<td>0.011</td>
<td>12.9</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 59/98/150</td>
<td>0.002</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Figure 6: The CAT Assay Curve of pKK232-8/BCG clone XIII/3

Notes: Chloramphenicol was added to the reaction mixture at $T = 300$ seconds.
8.3.3 Calculation of the Specific Activity of the Chloramphenicol Acetyltransferase Enzymes Expressed by the Twelve Clones

The specific activity of each of the twelve expressed chloramphenicol acetyltransferase (CAT) enzymes was calculated from the previously determined values for: (1) CAT activity (nmoles/min/ml), listed in Table 16 and (2) the total protein concentrations (mg/ml) of each of the twelve clones, listed in Table 15.

i.e. CAT specific activity = \( \frac{\text{CAT activity (nmoles/min/ml)}}{\text{(nmoles/min/mg)}} \times \frac{\text{total protein concentration (mg/ml)}}{\text{mg/ml}} \)

Table 17 lists the calculated specific activity values for the chloramphenicol acetyltransferase enzymes expressed by each of the twelve clones. Although there is some variation in the level of resistance per unit of enzyme, from one clone to another, the values are extremely consistent considering the range of resistance levels involved (i.e. 25µg/ml to 600µg/ml chloramphenicol for the pKK232-8/BCG clones and 25µg/ml to 800µg/ml chloramphenicol for the pKK232-8/HB101 clones). Thus, over a considerable range of resistance levels there is an approximately linear relationship between the amount of CAT expressed and the level of resistance to chloramphenicol achieved.
### Table 17: Specific Activity Values for the Twelve Expressed Chloramphenicol Acetyltransferase Enzymes

<table>
<thead>
<tr>
<th>Sample</th>
<th>CRL (mg/ml)*</th>
<th>CAT Specific Activities</th>
<th>CRL per unit of CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/min/mg</td>
<td>Enzyme units per mg total protein</td>
<td></td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/1</td>
<td>0.600</td>
<td>96.2</td>
<td>0.0962</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/3</td>
<td>0.500</td>
<td>71.2</td>
<td>0.0712</td>
</tr>
<tr>
<td>pKK232-8/BCG clone XIII/2</td>
<td>0.400</td>
<td>58.2</td>
<td>0.0582</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 87/4/160</td>
<td>0.100</td>
<td>15.6</td>
<td>0.0156</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 33/4/160</td>
<td>0.050</td>
<td>10.4</td>
<td>0.0104</td>
</tr>
<tr>
<td>pKK232-8/BCG clone 52/14/90</td>
<td>0.025</td>
<td>3.2</td>
<td>0.0032</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 66/3/150</td>
<td>0.800</td>
<td>171.0</td>
<td>0.1710</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone IX/1</td>
<td>0.600</td>
<td>116.4</td>
<td>0.1164</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 2/3/150</td>
<td>0.500</td>
<td>80.2</td>
<td>0.0802</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone III/1</td>
<td>0.400</td>
<td>79.6</td>
<td>0.0796</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 105/6/150</td>
<td>0.050</td>
<td>15.9</td>
<td>0.0159</td>
</tr>
<tr>
<td>pKK232-8/HB101 clone 59/98/150</td>
<td>0.025</td>
<td>3.2</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

Notes: CRL = Chloramphenicol resistance level

(*: previously, chloramphenicol resistance levels have been expressed in µg/ml)
Two plasmid vectors (pBR322 and pUC13) and two phage lambda vectors (EMBL4 and gtII) were employed in the production of \textit{M. bovis} BCG and \textit{M. leprae} gene libraries and short descriptions of some of the characteristic properties of these four cloning vectors are given below.

For a plasmid to be a useful cloning vector, suitable for the introduction of foreign DNA into a bacterial cell, a number of requirements need to be met: (1) the plasmid should be a small molecule (3-4Kb), as the efficiency of transformation of bacterial cells drops when large plasmids (c.15Kb) are used; (2) the plasmid should contain an origin of replication that operates in the host, i.e. the bacterial cell into which the plasmid is to be introduced; (3) the plasmid should contain at least one gene that can serve as a selectable marker for cells that have been transformed, e.g. a gene coding for antibiotic resistance and (4) the plasmid should contain one or more unique recognition sites for restriction enzymes (not located in essential regions of the plasmid), which may be used for the insertion of foreign DNA fragments.

The plasmid pBR322 (Figure 7), which is 4.3Kb in size was constructed from fragments originating from three naturally occurring plasmids (Bolivar et al., 1977 a; b) and contains two genes coding for antibiotic resistance: one gene encoding the production of beta-lactamase, confers ampicillin-resistance to the host cell and a second gene coding for tetracycline-resistance. Furthermore the plasmid
contains 20 unique restriction enzyme sites, six of which are located within the tetracycline-resistance gene (EcoRV, BamHI, SphI, SalI, XmaIII and NruI) and three of which lie within the ampicillin-resistance gene (PstI, PvuI and Scal) and the ligation of foreign DNA fragments into any of these nine sites results in the insertional inactivation of either one of the antibiotic resistance markers. Consequently, insertional inactivation aids the selection of bacterial cells carrying plasmids containing foreign DNA inserts.

The pUC series of plasmid vectors, developed by Vieira and Messing, are derivatives of pBR322 (containing the beta-lactamase gene but lacking the tetracycline-resistance gene), which contain a portion of the E.coli lacZ gene which encodes for the N-terminal alpha-peptide of the enzyme beta-galactosidase. When E.coli strains (e.g. JM103), producing defective beta-galactosidase are transformed with any of the pUC vectors, active beta-galactosidase is produced through complementation of the defective host-encoded enzyme and the defective vector-encoded enzyme. Furthermore, transformants containing the original, intact pUC plasmid can be selected as blue colonies on media containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). This is indicative of the formation of functional beta-galactosidase, capable of hydrolysing X-gal and producing a blue dye (which then stains the colony). Situated within the lacZ portion of each pUC vector is an array of unique restriction enzyme sites (each of the vectors in the pUC series differs in the number and orientation of these restriction sites), pUC13 (Figure 8), the pUC vector used in this study contains unique restriction sites for
the following twelve enzymes HindIII, PstI, SalI, AccI, HincII, XbaI, BamHI, XmaI, SmaI, SstI, EcoRI and HaeIII. Insertion of foreign DNA into any one of these restriction sites results in the loss of the activity of the expressed alpha-peptide, thus functional beta-galactosidase is not produced and subsequently recombinant transformants are detected as white colonies on media containing X-gal. Furthermore, DNA fragments ligated into any of the twelve restriction sites described above are placed under the control of the lacZ promoter and have the potential to be expressed as fusion proteins with the N-terminal portion of the lacZ gene.

Figure 7: Simplified Restriction Map of the Plasmid pBR322

Notes: amp = ampicillin-resistance gene; tet = tetracycline-resistance gene; the arrows indicate the direction in which the antibiotic resistance genes are transcribed.
Figure 8: Simplified Restriction Map of the Plasmid pUC13

Notes: amp = ampicillin-resistance gene; lacZα = codes for the alpha-peptide of beta-galactosidase; the arrows indicate the direction in which the genes are transcribed.
Wild-type phage lambda is unsuitable as a cloning vector due to limitations in the amount of DNA which can be packaged into phage lambda particles and still result in the formation of viable phage. The lower packaging limit is 80% of the normal length of the wild-type phage lambda genome and constitutes the minimum amount of phage lambda DNA needed to code for the genes essential for lytic growth; the upper packaging limit corresponds to the maximum amount of DNA that is packageable in phage lambda heads and is calculated to be 105% of the normal length of the wild-type phage lambda genome. The unsuitability of wild-type phage lambda as a cloning vector is further emphasised by the presence of several restriction sites, for most of the commonly used restriction enzymes, within its 48.5Kb genome. Fortunately, derivatives of wild-type phage lambda have been constructed and some are able to accept large fragments of foreign DNA. Two types of phage lambda vectors have been constructed: (1) replacement vectors, which contain a pair of restriction sites at either side of a non-essential region (stuffer fragment) of the phage lambda genome, allowing the stuffer fragment to be removed and replaced with a foreign DNA fragment and (2) insertion vectors, which contain a unique cloning site, within a non-essential region of the phage lambda genome, into which foreign DNA can be ligated.

The phage lambda vector EMBL4 (Frischauf et al., 1983) is a replacement vector containing EcoRI, BamHI and SalI restriction sites at either side of its stuffer fragment (Figure 9). The subsequent removal of this stuffer fragment (digestion of the vector with one of the three enzymes described above) allows foreign DNA fragments of 9-23Kb to be
ligated into this vector. Another feature of this vector is the presence of two particular genes, i.e. red and gam genes, whose phenotypes can be used in the selection of recombinant molecules (i.e. containing foreign DNA inserts). The products of these two genes are responsible for inhibiting growth of EMBL4 phage on E.coli strains lysogenic for the phage P2, i.e. EMBL4 is Spi⁻ (sensitive to E2 inhibition). The red and gam genes are located within the EMBL4 stuffer fragment, consequently EMBL4 recombinants are Spi⁻ and can be directly selected by using an E.coli strain lysogenic for the phage P2, as the infection host (e.g. E.coli WM539). The EMBL4 vector employed in this thesis was purchased from Amersham International as EMBL4 Arms. The manufacturers had digested the vector with BamHI and then removed the stuffer fragment.

The phage lambda vector gt11 (Young and Davis, 1983a;b) is an insertion vector that permits the ligation of up to 7Kb of foreign DNA into a unique EcoRI cloning site (Figure 10). Furthermore, this vector also contains the E.coli lacZ gene (coding for functional beta-galactosidase) and it is within this gene that the EcoRI cloning site is located. Foreign DNA fragments ligated into this site are under the control of the lacZ promoter and are subsequently inducible with IPTG (isopropyl-beta-D-thio-galactopyranoside) moreover, these foreign DNA fragments have the potential to be expressed as fusion proteins with beta-galactosidase. The insertion of foreign DNA within the lacZ gene inactivates the beta-galactosidase enzyme and consequently recombinant gt11 molecules can be selected by their inability to produce blue plaques when plated out onto a lawn of E.coli lac⁻ cells, on media
containing X-gal. As the foreign DNA fragments are under the control of an *E. coli* promoter, this cloning system ensures that these fragments will be expressed efficiently in *E. coli* moreover, the resultant fusion proteins will be highly stable.

**Figure 9:** Simplified Restriction Map of Phage Lambda Vector EMBL4

Notes:  
- R = EcoRI restriction site;  
- B = BamHI;  
- S = SalI;  
- Left arm = 19.3Kb;  
- Stuffer fragment = 13.7Kb;  
- Right arm = 9.3Kb.
Figure 10: Simplified Restriction Map of Phage Lambda Vector gt11

Notes: The arrow indicates the direction in which the lacZ gene is transcribed. The sequence of the unique EcoRI cloning site and the nucleotides that immediately surround it are also shown.
9.1 Cloning \textit{M. bovis} BCG DNA into the Plasmid Vector pBR322

\textit{M. bovis} BCG DNA (c.8μg) was partially digested with BamHI for 20 minutes. The restriction fragments were then size-fractionated on an agarose gel and appropriate sized fragments (2–9Kb) were recovered and ligated with pBR322, which had been digested with BamHI and then CIAP-treated. Table 18 below, describes the preparation of the pBR322/\textit{M. bovis} BCG ligation mixture, together with two control mixtures. The three ligation mixtures were then incubated at 12°C overnight.

\textbf{Table 18: Ligation Mixture and Controls: Ligating \textit{M. bovis} BCG DNA Restriction Fragments into the Plasmid pBR322}

<table>
<thead>
<tr>
<th>Ligation Mixture (μl)</th>
<th>Controls (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322, BamHI-digested, CIAP treated</td>
<td>20 (2μg)</td>
</tr>
<tr>
<td>\textit{M. bovis} BCG DNA, BamHI digested</td>
<td>40 (3μg)</td>
</tr>
<tr>
<td>pBR322, uncut</td>
<td>-</td>
</tr>
<tr>
<td>5mM ATP</td>
<td>10</td>
</tr>
<tr>
<td>10X Ligation buffer</td>
<td>10</td>
</tr>
<tr>
<td>T4-DNA Ligase</td>
<td>5</td>
</tr>
<tr>
<td>distilled water</td>
<td>15</td>
</tr>
</tbody>
</table>
Following overnight incubation, the pBR322/M. bovis BCG ligation mixture and the two control mixtures were used to transform competent *E. coli* HB101 cells. Transformation mixtures were prepared (described below) and subjected to the transformation procedure outlined in section 6.6.2.

**Transformation mixtures:**

A = Control No. 1 + 200µl competent cells  
B = Control No. 2 + 200µl competent cells  
C = Ligation mixture: 
   pBR322/M. bovis BCG (x20) + 200µl competent cells (x20)

Following transformation, the mixtures were centrifuged and the pellets were resuspended in 0.1ml volumes of L-Broth, then plated out onto L-Agar plates containing ampicillin (50µg/ml). The plates were then incubated at 37°C overnight.

Table 19 lists the total number of ampicillin-resistant transformants obtained following (1) the pBR322/M. bovis BCG cloning experiment and (2) the two control experiments. The result of Control No. 2 implies that the preparation of the vector had been very good and it was anticipated that a high proportion of the c.9000 pBR322/M. bovis BCG transformants would be recombinant.

The BamHI cloning site of pBR322 is located within the tetracycline-resistance gene, allowing recombinant transformants to be
detected by their inability to grow on media containing tetracycline. Accordingly, 202 ampicillin-resistant pBR322/M. bovis BCG transformants were randomly selected and tested for tetracycline-resistance, on L-Agar plates containing 10μg/ml tetracycline. Only nine ampicillin-resistant, tetracycline-resistant transformants were detected, consequently the c.9000 clone pBR322/M. bovis BCG library was calculated to be c.95% recombinant.

The c.9000 pBR322/M. bovis BCG clones were pooled and amplified, according to the method described in section 6.6.3. The titre of the amplified library was subsequently calculated to be $1.5 \times 10^8$ clones/ml.

Table 19: The Numbers of Ampicillin-Resistant Colonies: Cloning M. bovis BCG DNA into pBR322

<table>
<thead>
<tr>
<th>Cloning Experiment</th>
<th>Ampicillin-Resistant Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322, BamHI-digested, CIAP-treated + M. bovis BCG DNA, BamHI-digested</td>
<td>c.9000</td>
</tr>
<tr>
<td>Controls:</td>
<td></td>
</tr>
<tr>
<td>1: pBR322, uncut</td>
<td>Uncountable</td>
</tr>
<tr>
<td>2: pBR322, BamHI-digested, CIAP-treated + ligase</td>
<td>5</td>
</tr>
</tbody>
</table>
2.2 Cloning M. leprae DNA into the Plasmid Vector pBR322

Two aliquots of M. leprae DNA (500ng each) were partially digested with (1) BamHI and (2) Sau3A, for 30 minutes. Due to the very small amounts of M. leprae DNA in the two digestion mixtures, it was considered unwise to attempt to size-fractionate the digests, to selectively recover restriction fragments 2-9Kb in size, for ligating into pBR322; DNA losses would have been incurred during (i) the recovery of the restriction fragments from the agarose gel and (ii) the ethanol precipitation step (see method, section 6.5.6). Consequently, following digestion the two digests were heated at 70°C for 10 minutes, to inactivate their respective restriction enzymes and then aliquots of each digest were ligated into the BamHI site of pBR322 (Sau3A restriction fragments can be ligated with BamHI digested pBR322 as their cohesive ends are identical), as described in section 6.6.1. Two control ligations were also prepared (1) pBR322 uncut and (2) pBR322, BamHI-digested, CIAP-treated + ligase, as described in Table 18.

Following overnight incubation at 12°C, the pBR322/M. leprae ligation mixtures and the two control mixtures were used to transform competent E. coli HB101 cells, according to the procedure described in section 6.6.2. Following transformation, aliquots of the transformation mixtures were plated out onto L-Agar plates containing ampicillin and these plates were then incubated at 37°C overnight.

Table 20 lists the total number of ampicillin-resistant transformants obtained from (1) the two pBR322/M. leprae cloning
experiments and (2) the two control experiments. The result of Control
No. 2 implies that the preparation of the vector had been very good;
the result of Control No. 1 implies that competent cells had been
produced and that the transformation reaction worked, thus it was
anticipated that a high proportion of the pBR322/M.leprae transformants
obtained, would be recombinant.

A number of ampicillin-resistant pBR322/M.leprae transformants
were selected and tested for insertional inactivation of the
tetracycline-resistance gene, i.e. 241 pBR322/M.leprae (BamHI fragments)
transformants and 334 pBR322/M.leprae (Sau3A fragments) transformants,
were tested for growth on L-Agar plates containing 10μg/ml tetracycline.
Subsequently, 91% of the pBR322/M.leprae (BamHI) transformants and 97%
of the pBR322/M.leprae (Sau3A) transformants were determined to be
recombinant. The small number of clones in the two pBR322/M.leprae
libraries, allowed each clone to be stored separately, in 100μl volumes
of L-Broth. (A pooled stock of the 2497 transformants was also prepared
and amplified).
### Table 20: The Numbers of Ampicillin-Resistant Colonies: Cloning M. leprae DNA into pBR322

<table>
<thead>
<tr>
<th>Cloning Experiment</th>
<th>Ampicillin-Resistant Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322, BamHI-digested, CIAP-treated + M. leprae DNA, BamHI-digested</td>
<td>1286</td>
</tr>
<tr>
<td>pBR322, BamHI-digested, CIAP-treated + M. leprae DNA, Sau3A-digested</td>
<td>1211</td>
</tr>
<tr>
<td>Controls:</td>
<td></td>
</tr>
<tr>
<td>1: pBR322, uncut</td>
<td>594</td>
</tr>
<tr>
<td>2: pBR322, BamHI-digested, CIAP-treated + ligase</td>
<td>8</td>
</tr>
</tbody>
</table>
2.3 Cloning M. leprae DNA into the Plasmid Vector pUC13

An aliquot of M. leprae DNA was partially digested with Sau3A for 30 minutes and then ligated (as described in section 6.6.1) with pUC13, which had been digested with BamHI then CIAP-treated. Two control ligations were also prepared (see below) which, together with the pUC13/M. leprae ligation mixture, were incubated at 12°C overnight.

Control Ligations:

No. 1: 50ng pUC13 DNA, uncut + 2μl 5mM ATP + 2μl 10X Ligation buffer + 15μl distilled water.

No. 2: 50ng pUC13 DNA, BamHI-digested, CIAP-treated + 2μl 5mM ATP + 2μl 10X Ligation buffer + 1 unit T4-DNA ligase + 14μl distilled water.

Following overnight incubation at 12°C, the pUC13/M. leprae ligation mixture and the two control mixtures were used to transform competent E. coli JM103 cells, in accordance with the procedure outlined in section 6.6.2. Aliquots of the transformation mixtures were then plated out onto L-Agar plates containing ampicillin (50μg/ml), 10mM IPTG and 0.2% X-gal, which were then incubated at 37°C overnight.

When plated out onto media containing the substrate X-gal, non-recombinant transformants are detected as blue colonies, indicative of the formation of a functional beta-galactosidase enzyme capable of hydrolysing X-gal and producing a blue dye (i.e. the beta-galactosidase,
alpha-peptide expressed by pUC13 complements the defective enzyme produced by the JM103 cells, producing a functional enzyme); recombinant transformants are white (ligation of DNA into the pUC13 BamHI site results in the expression of an inactive beta-galactosidase, alpha-peptide, unable to complement the defective enzyme produced by the JM103 cells).

Table 21 lists the total number of ampicillin-resistant transformants, obtained following: (1) the pUC13/M. leprae cloning experiment and (2) the two control experiments. The result of Control No. 2 suggested that the preparation of the vector had been very good. Consequently, it had been anticipated that a greater percentage of white colonies (recombinant transformants) would be produced by the pUC13/M. leprae cloning experiment, than was actually achieved. However, providing a cloned M. leprae DNA fragment was small and maintained the reading frame of the pUC13 lacZ gene, a functional alpha-peptide could still be expressed by the pUC13 vector able to complement the defective beta-galactosidase enzyme of the host cell (E. coli JM103) and form an active enzyme capable of hydrolysing X-gal and producing a blue colony. Thus some of the 1732 blue colonies produced by the pUC13/M. leprae cloning experiment may be recombinants.
Table 21: The Numbers of Ampicillin-Resistant Colonies: Cloning M. leprae DNA into pUC13

<table>
<thead>
<tr>
<th>Cloning Experiment</th>
<th>Ampicillin-Resistant Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
</tr>
<tr>
<td>pUC13, BamHI-digested, CIAP-treated + M. leprae DNA, Sau3A-digested</td>
<td>2046</td>
</tr>
</tbody>
</table>

Controls:

1: pUC13, uncut | 0 | Uncountable |
2: pUC13, BamHI-digested, CIAP-treated + ligase | 0 | 3 |
9.4 Cloning *M. bovis* BCG DNA into the Phage Lambda Vector EMBL4

*M. bovis* BCG DNA (c.2µg) was partially digested with BamHI for 20 minutes and the restriction fragments were then size-fractionated on an agarose gel. Appropriate sized fragments (larger than 9Kb in size) were recovered from the gel and ligated with the BamHI arms of the phage lambda vector EMBL4, as described in section 6.7.1.

The EMBL4/M. bovis BCG ligation mixture was then subjected to in vitro packaging (section 6.7.3) and the resultant packaging mixture constituted the EMBL4/M. bovis BCG library. To determine the number of recombinant molecules in the library, 200µl aliquots of *E. coli* WM539 cells were infected with diluted aliquots of the EMBL4/M. bovis BCG packaging mixture (as described in section 6.7.4) and these infection mixtures were then plated out onto L-Agar plates, then incubated at 37°C overnight. The numbers of plaques on each plate were counted (Table 22 lists the results of the infection experiments) and the number of recombinant molecules in the unamplified EMBL4/M. bovis BCG library was determined from the following calculations:

1. 140 plaques were obtained when 100µl of a 10⁻² dilution of the unamplified library was plated onto an L-Agar plate (Plate 1; see Table 22), implying (i) c.1400 plaques would be obtained if 1ml of a 10⁻² dilution of the library were plated onto an L-Agar plate and (ii) c.1.4 x 10⁸ plaques would be obtained if 1ml of the undiluted, unamplified library were plated out onto an L-Agar plate.
(2) As the total volume of the unamplified EMBL4/M. bovis BCG library was only 0.5ml, the library was thus calculated to contain c.7 x 10^4 recombinant molecules (i.e. 1.4 x 10^5 x 0.5).

The library was amplified as described in section 6.7.5 and the titre of the amplified EMBL4/M. bovis BCG library was determined to be c. 1 x 10^8 recombinant molecules/ml.

Table 22: Cloning M. bovis BCG DNA into the Phage Lambda Vector
EMBL4: Determining the Size of the EMBL4/M. bovis BCG Library

<table>
<thead>
<tr>
<th>Plate</th>
<th>Packaging Mixture Dilution</th>
<th>Plaque Numbers</th>
<th>EMBL4/M. bovis BCG Library size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100μls 10^-2 dilution EMBL4/M. bovis BCG Packaging Mixture (+200μls NM539 cells)</td>
<td>140</td>
<td>c.7 x 10^4 recombinant molecules</td>
</tr>
<tr>
<td>2</td>
<td>300μls 10^-2 dilution EMBL4/M. bovis BCG Packaging Mixture (+200μls NM539 cells)</td>
<td>396</td>
<td></td>
</tr>
</tbody>
</table>
9.5 Cloning *M. bovis* BCG and *M. leprae* DNA into the Phage Lambda Vector gtII

In preparation for their subsequent ligation with phage lambda vector gtII, aliquots of *M. bovis* BCG and *M. leprae* DNA were each mechanically sheared by 225 passages through a 25-gauge needle (section 6.7.2). The sheared fragments were then size-fractionated on an agarose gel and fragments up to 7Kb in size were selectively recovered.

Following the method in section 6.7.2, the EcoRI restriction sites within the sheared DNA fragments were methylated and the fragments were then blunt-ended. EcoRI linkers were ligated to the blunt ended *M. bovis* BCG and *M. leprae* DNA fragments and these fragments were then ligated into the EcoRI site of gtII.

Following their incubation at 12°C overnight, the gtII/*M. bovis* BCG and the gtII/*M. leprae* ligation mixtures were subjected to in vitro packaging (section 6.7.3) and the resultant packaging mixtures constituted the gtII/*M. bovis* BCG and gtII/*M. leprae* libraries. To determine the numbers of recombinant molecules in each phage library, 200μl aliquots of *E. coli* Y1088 cells were infected with diluted aliquots of the gtII packaging mixtures (as described in section 6.7.4) and these infection mixtures were then plated out onto L-Agar plates containing ampicillin (50μg/ml), IPTG (10mM) and X-gal (0.2%). Following incubation of the plates, at 42°C overnight, the numbers of clear plaques (recombinant clones) and blue plaques (non-recombinant clones) on each plate were counted (see Table 23) and from these results, the
gtII/M._bovis_ BCG library was calculated to contain c.1 x 10^6 recombinant clones and (2) the gtII/M._leprae_ library contained c.4 x 10^4 recombinant clones.

Both libraries were amplified as described in section 6.7.5. Assaying the amplified libraries subsequently revealed that (i) the titre of the amplified gtII/M._bovis_ BCG library was c.4 x 10^10 phage/ml moreover, 91% of the molecules were recombinant and (ii) the titre of the amplified gtII/M._leprae_ library was c.7 x 10^10 phage/ml, with 40% of phage recombinant. Not all recombinants in a population will propagate equally well, e.g. variations in insert size or sequence may affect replication of a recombinant molecule. Therefore, when a library is amplified, poorly-growing recombinants may be decreased in frequency or lost altogether, which would account for the slight fall in the % of recombinants observed following amplification of the two libraries.
Table 23: Cloning M. bovis BCG and M. leprae DNA into the Phage Lambda Vector gtII: Determining the Size of the Two Libraries

<table>
<thead>
<tr>
<th>Packaging Mixture Dilution</th>
<th>Plaque Numbers</th>
<th>Percentage Recombinant Phage per Library</th>
<th>Library Size (Recombinant Molecules)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10μl 10⁻² dilution gtII/ M. bovis BCG Packaging Mixture (+200μl Y1088 cells)</td>
<td>230 Clear, 13 Blue</td>
<td>95%</td>
<td>c.1 x 10⁶</td>
</tr>
<tr>
<td>10μl 10⁻¹ dilution gtII/ M. leprae Packaging Mixture (+200μl Y1088 cells)</td>
<td>82 Clear, 56 Blue</td>
<td>60%</td>
<td>c.4 x 10⁴</td>
</tr>
</tbody>
</table>
Using rabbit anti-	extit{M. bovis} BCG antiserum (as the primary antibody), together with a number of secondary antibody solutions (e.g. horseradish peroxidase conjugated goat anti-rabbit IgG; biotin conjugated goat anti-rabbit IgG; alkaline phosphatase conjugated goat anti-rabbit IgG), the \textit{M. bovis} BCG gene libraries, i.e. pBR322/\textit{M. bovis} BCG, EMBL4/\textit{M. bovis} BCG and gtII/\textit{M. bovis} BCG were screened to identify clones expressing \textit{M. bovis} BCG antigens.

10.1 In Situ Screening of the pBR322/\textit{M. bovis} BCG Library

Diluted aliquots of the pBR322/\textit{M. bovis} BCG library were prepared and plated out onto 15 L-Agar plates. Following overnight incubation at 37°C, each plate (containing c.500 colonies) was overlaid with a nitrocellulose filter disc and the plates + filters were then re-incubated at 37°C for a further 5 minutes. The filters were removed from the plates and those cells which had transferred to the filters, were lysed in accordance with the method described in section 6.9.

Following treatment to block their non-specific protein binding sites, the 15 filters were then incubated in (1) a 1:500 dilution of an \textit{E. coli} HB101 absorbed rabbit anti-	extit{M. bovis} BCG antiserum solution, prepared as described in section 6.8 and then in (2) a 1:3000 dilution of a horseradish peroxidase conjugated goat anti-rabbit IgG, second antibody solution, as described in section 6.9. Following incubation of
the filters in the horseradish peroxidase colour development solution, a
total of 19 positive signals were detected.

The clones corresponding to the positive signals were recovered
from the appropriate L-Agar plates, overnight cultures of each clone
were prepared and then an aliquot of each culture was analysed by SDS-
polyacrylamide gel electrophoresis (SDS-PAGE), as described in section
6.13.1, in order to identify the *M. bovis* BCG proteins expressed by each
of the selected pBR322/*M. bovis* BCG clones.

Following electrophoresis the, SDS-PAGE gel was stained (PAGE
Blue 83) and then destained as described in section 6.14 and numerous
protein bands were subsequently detected in all of the tracks of the
gel. Unfortunately, the protein banding patterns of all the 19
recombinant samples and the *E. coli* HB101 control sample appeared
identical and it was not possible therefore, to identify expressed
*M. bovis* BCG protein bands.

To overcome limitations in the sensitivity and the discrimina-
tion of protein stains, the 19 pBR322/*M. bovis* BCG clones were
electrophoresed through a second SDS-PAGE gel, which was then Western
blotted and the resultant nitrocellulose filter was screened with rabbit
anti-*M. bovis* BCG antibodies, as described in section 6.13.3.

Western blot analysis revealed that all 19 of the pBR322/*M. bovis*
BCG clones were expressing three antigenic protein bands, all of which
corresponded to the proteins expressed by the *E. coli* control sample.
Furthermore, two of the 19 clones (i.e. pBR322/M. bovis clones A and E) were also shown to be expressing unique protein bands (i.e. antigenic M. bovis BCG proteins) which did not have corresponding counterparts in the E. coli control sample (Photograph 2). Further analysis of these two clones, by SDS-PAGE electrophoresis and Western blotting, revealed that both pBR322/M. bovis BCG clone A and pBR322/M. bovis BCG clone E were expressing a 65KD M. bovis BCG antigenic protein (Photograph 3).
Photograph 2: Western Blot Analysis of Fourteen pBR322/M.bovis BCG Clones

Track number
1 = E.coli HB101 (Control)
2 = pBR322/M.bovis BCG clone A
3 = pBR322/M.bovis BCG clone B
4 = pBR322/M.bovis BCG clone C
5 = pBR322/M.bovis BCG clone D
6 = pBR322/M.bovis BCG clone E
7 = pBR322/M.bovis BCG clone F
8 = pBR322/M.bovis BCG clone G
9 = pBR322/M.bovis BCG clone H
10 = pBR322/M.bovis BCG clone J
11 = pBR322/M.bovis BCG clone K
12 = pBR322/M.bovis BCG clone L
13 = pBR322/M.bovis BCG clone M
14 = pBR322/M.bovis BCG clone N
15 = pBR322/M.bovis BCG clone P
Following Western blotting, the nitrocellulose filter was screened with:

1. a 1:500 dilution of *E. coli* HB101 absorbed rabbit anti-*M. bovis* BCG antiserum;
2. a 1:3000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG solution and then developed in the horseradish peroxidase colour development solution.
Photograph 3: Size Analysis of the M.bovis BCG Proteins Expressed by pBR322/M.bovis BCG Clones A and E

Track number

1  pBR322/M.bovis BCG Clone A
2  pBR322/M.bovis BCG Clone E
10.1.1 Determining the Relationship Between the 65KD Antigenic M. bovis
BCG Proteins Expressed by pBR322/M. bovis BCG Clones A and E

In an attempt to determine whether pBR322/M. bovis BCG clones A
and E were expressing the same 65KD antigenic protein, two aliquots of
each clone were subjected to SDS-PAGE electrophoresis (aliquots of clone
A were loaded into tracks 1 and 5; aliquots of clone E were loaded
into tracks 2 and 6) and the gel was then Western blotted, as described
previously. Following blotting the nitrocellulose filter was cut into
two fragments (Filter I: tracks 1-4; Filter II: tracks 5-8). Filter I
was then screened with E. coli HB101 absorbed rabbit anti-M. bovis BCG
antiserum and Filter II with pBR322/M. bovis BCG clone A absorbed rabbit
anti-M. bovis BCG antiserum (this antiserum solution was prepared in
accordance with the method described in section 6.8). Both filters were
then screened with (i) biotin conjugated secondary antibodies and (ii) a
streptavidin-biotinylated horseradish peroxidase solution.

Following their incubation in the horseradish peroxidase colour
development solution, differences were detected in the banding patterns
of the two filters. (Photograph 4). The intensity of the 65KD
antigenic M. bovis BCG protein expressed by clone A was stronger on
Filter I (track 1) than on Filter II (track 5) and this result confirmed
that antibodies against the 65KD M. bovis BCG protein expressed by clone
A, had been absorbed from the antiserum solution used to screen Filter
II, as intended. Moreover, the intensity of the 65KD antigenic M. bovis
BCG protein expressed by clone E was also stronger on Filter I (track 2)
than on Filter II (track 6) and this result suggested that antibodies
against the 65KD *M. bovis* BCG protein expressed by clone E had also been removed from the absorbed antiserum solution used to screen Filter II. This result implied that pBR322/*M. bovis* BCG clone A and pBR322/*M. bovis* BCG clone E were both expressing the same 65KD antigenic *M. bovis* BCG protein.
Photograph 4: Screening the 65KD Antigenic M. bovis BCG Proteins Expressed by pBR322/M. bovis BCG clones A and E in the Presence and Absence of an Absorbed Antiserum Solution

Track number

1, 5 = pBR322/M. bovis BCG clone A
2, 6 = pBR322/M. bovis BCG clone E
3, 7 = M. bovis BCG
4, 8 = E. coli HB101

FILTER I

1 2 3 4

FILTER II

5 6 7 8

Filter I: screened with (i) a 1:500 dilution of E. coli HB101 absorbed rabbit anti-M. bovis BCG antiserum; (ii) a 1:2000 dilution of biotin conjugated goat anti-rabbit IgG and (iii) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution.

Filter II: screened with (i) a 1:50 dilution of pBR322/M. bovis BCG clone A absorbed rabbit anti-M. bovis BCG antiserum; (ii) a 1:2000 dilution of biotin conjugated goat anti-rabbit IgG and (iii) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution.
10.1.2 Restriction Enzyme Analysis of pBR322\_M.\_bovis BCG Clones A and E

Plasmid DNA was prepared for pBR322\_M.\_bovis BCG clones A and E, according to the method described in section 6.3.1 and aliquots of each were subsequently digested with the restriction enzyme BamHI. Analysis of both digests by agarose gel electrophoresis revealed six BamHI restriction fragments for pBR322\_M.\_bovis BCG clone A and four BamHI restriction fragments for pBR322\_M.\_bovis BCG clone E (Photograph 5), and the sizes of these BamHI restriction fragments are listed below:

(1) pBR322\_M.\_bovis BCG clone A x BamHI - six restriction fragments

- 4.3Kb
- 2.5Kb
- 1.8Kb
- 1.3Kb
- 0.9Kb
- and 0.8Kb

(2) pBR322\_M.\_bovis BCG clone E x BamHI - four restriction fragments

- 4.3Kb
- 1.8Kb
- 1.2Kb
- and 0.9Kb

From the above results, the two clones were shown to share three BamHI restriction fragments: a 4.3Kb fragment (linearised pBR322); a 1.8Kb fragment and a 0.9Kb fragment. This result further suggests that the two clones are expressing the same 65KD antigenic protein. However, the difference in the size of their respective inserts (pBR322\_M.\_bovis BCG
clone A's insert was calculated to be 7.3Kb; *pBR322/M.bovis* BCG clone E's insert was only 3.9Kb) suggests that clones A and E are probably not re-isolations of the same clone.

From the information obtained from a series of single and double digests of *pBR322/M.bovis* BCG clone A DNA and from the known restriction map of pBR322, a partial restriction map was constructed for the 7.3Kb insert of *pBR322/M.bovis* BCG clone A (Figure 11). Table 24 lists details of the digests which were performed and the number and sizes of the restriction fragments produced.
Photograph 5: Restriction Enzyme Analysis of \text{pBR322/M. bovis BCG Clones A}

and E

Track number

1 = BamHI digested \text{pBR322/M. bovis BCG clone A}
2 = BamHI digested \text{pBR322/M. bovis BCG clone E}
3 = EcoRI digested \text{pBR322/M. bovis BCG clone A}
4 = EcoRI digested \text{pBR322/M. bovis BCG clone E}
5 = HindIII digested Lambda DNA
6 = HaeIII digested \text{\phi X174}
Table 24: Restriction Analysis of pBR322/M.bovis BCG Clone A

<table>
<thead>
<tr>
<th>Restriction Digest</th>
<th>Number of Sites within pBR322</th>
<th>Number of Restriction Fragments produced</th>
<th>Sizes of the Restriction Fragments (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>1</td>
<td>6</td>
<td>4.3, 2.5, 1.8, 1.3 0.9, 0.8</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1</td>
<td>2</td>
<td>c.9.0, 2.3</td>
</tr>
<tr>
<td>KpnI</td>
<td>0</td>
<td>2</td>
<td>c.10.0, 1.4</td>
</tr>
<tr>
<td>Sall</td>
<td>1</td>
<td>6</td>
<td>6.0, 3.5, 0.75 0.55, 0.5, 0.3</td>
</tr>
<tr>
<td>PstI</td>
<td>1</td>
<td>3</td>
<td>c.9.0, 1.9, 1.25</td>
</tr>
<tr>
<td>HindIII</td>
<td>1</td>
<td>1</td>
<td>11.6</td>
</tr>
<tr>
<td>HindIII/PstI</td>
<td>2</td>
<td>4</td>
<td>c.8.0, 1.25, 1.15 0.8</td>
</tr>
<tr>
<td>BamHI/KpnI</td>
<td>1</td>
<td>8</td>
<td>4.3, 2.1, 1.3, 1.0 0.9, 0.8, 0.8, 0.4</td>
</tr>
<tr>
<td>BamHI/EcoRI</td>
<td>2</td>
<td>8</td>
<td>4.0, 2.5, 1.3, 1.1 0.9, 0.8, 0.7, 0.3</td>
</tr>
</tbody>
</table>
Figure 11: Partial Restriction Map of the pBR322/X. hovis BCG clone A

Insert

Notes: B = BamHI; R = EcoRI; S = SalI; P = PstI; K = KpnI
10.2 Comparison of the 65KD Antigenic M. bovis BCG Protein Expressed by pBR322/M. bovis BCG clone A with 65KD Proteins Expressed by Other Mycobacterial Species

According to the literature, three groups had cloned 64-65KD proteins from four different mycobacterial species: Young et al. (1985 a; b) described recombinant gtII clones expressing (i) a 65KD antigenic M. tuberculosis protein and (ii) a 65KD antigenic M. leprae protein; Thole et al. (1985) described an EMBL3 clone expressing a 64KD antigenic M. bovis BCG protein and Labidi et al. (1985) described a pBR322 clone expressing a 64KD M. fortuitum protein. I subsequently obtained samples of three of these recombinant clones: (i.e. clone Y3178: expressing the 65KD M. leprae protein; clone pRIB1000: a plasmid clone expressing the 64KD M. bovis BCG protein and clone pAL15: expressing the 64KD M. fortuitum protein) and through the experiments described below, attempted to determine the extent of cross-reactivity between each of these 64-65KD mycobacterial proteins and the 65KD M. bovis BCG protein expressed by pBR322/M. bovis BCG clone A.

10.2.1 Analysis of the 64-65KD Mycobacterial Proteins with an Anti-M. bovis BCG Antiserum

Samples of the recombinant plasmid clones pBR322/M. bovis BCG clone A, pRIB1000 and pAL15 were prepared as described in section 6.13.1 and a crude protein extract of the phage lambda clone Y3178 was prepared in accordance with the procedure outlined in section 6.12. Two aliquots of each of the four samples were then loaded onto an SDS-PAGE gel:
pBR322/\textit{M. bovis} BCG clone A (tracks 1 and 5); pRIB1000 (tracks 2 and 6); Y3178 (tracks 3 and 7) and pAL15 (tracks 4 and 8), which was then subjected to electrophoresis (section 6.13.1). Following electrophoresis the gel was Western blotted and the resultant nitrocellulose filter was then cut into two fragments (Filter I: tracks 1-4; Filter II: tracks 5-8).

Filter I was screened with \textit{E. coli} HB101 absorbed rabbit anti-\textit{M. bovis} BCG antiserum and Filter II with pBR322/\textit{M. bovis} BCG clone A absorbed rabbit anti-\textit{M. bovis} BCG antiserum. Both filters were then screened with alkaline phosphatase conjugated secondary antibodies and finally the filters were incubated in the phosphatase colour development solution (Photograph 6).

Development of Filter I revealed antigenic bands of c.65KD in tracks 1, 2 and 3 (marked with —*—), thus showing that the 64-65KD mycobacterial proteins expressed by pBR322/\textit{M. bovis} BCG clone A, clone pRIB1000 and clone Y3178 were all cross-reactive with anti-\textit{M. bovis} BCG antibodies (not surprising results for clones A and pRIB1000, as these clones express \textit{M. bovis} BCG antigens). The 64KD \textit{M. fortuitum} protein (expressed by clone pAL15) was however, not detected on Filter I (track 4), implying that this protein is (i) not cross-reactive with anti-\textit{M. bovis} BCG antibodies and consequently (ii) not related to the 64-65KD \textit{M. bovis} BCG and \textit{M. leprae} proteins expressed by pBR322/\textit{M. bovis} BCG clone A, pRIB1000 and clone Y3178. In addition to the 64KD \textit{M. bovis} BCG protein, a number of lower molecular weight, antigenic bands were also
detected in track 2 (60-29KD in size) however, these were simply degradation products of the 64KD antigenic protein (see below).

Comparison of Filters I and II revealed that the intensities of the 64-65KD antigenic proteins (expressed by pBR322/M. bovis BCG clone A, clone pRIB1000 and clone Y3178) were all weaker on Filter II than on Filter I. These results implied that the antibodies to the (i) 64KD M. bovis BCG protein expressed by clone pRIB1000 and (ii) 65KD M. leprae protein expressed by clone Y3178, had been removed from the antiserum solution used to screen Filter II (the antiserum solution used to screen Filter II had been absorbed to remove antibodies to the 65KD M. bovis BCG antigenic protein expressed by pBR322/M. bovis BCG clone A). Furthermore, the lower molecular weight antigenic protein bands (60-29KD), seen in track 2 (Filter I), were not detected in track 6 (Filter II), confirming that these bands were degradation products of the 64KD M. bovis BCG protein expressed by clone pRIB1000. These results confirmed that (i) the 65KD M. bovis BCG proteins expressed by pBR322/M. bovis BCG clone A and clone pRIB1000 are the same M. bovis BCG protein and (ii) antibodies to the 65KD M. bovis BCG protein are cross-reactive with the 65KD M. leprae protein expressed by clone Y3178, thus the 65KD M. bovis BCG protein and the 65KD M. leprae protein must share common epitopes, which suggests that these two 65KD, mycobacterial proteins are homologues.
Photograph 6: Recombinant Mycobacterial Proteins Screened with a Rabbit Anti-M. bovis BCG Antiserum

Track numbers

1, 5 = pBR322/M. bovis BCG clone A
2, 6 = clone pRIB1000
3, 7 = clone Y3178
4, 8 = clone pAL15

Filter I: screened with (i) a 1:500 dilution of E. coli HB101 absorbed rabbit anti-M. bovis BCG antiserum and (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG.

Filter II: screened with (i) a 1:50 dilution of pBR322/M. bovis BCG clone A absorbed rabbit anti-M. bovis BCG antiserum and (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG.
10.2.2 Analysis of the 64-65KD Mycobacterial Proteins with an Anti-65KD M. leprae Monoclonal Antibody

Protein extracts of the four recombinant clones: pBR322/M. bovis BCG clone A (track 1), clone pRIB1000 (track 2), clone Y3178 (track 3) and clone pAL15 (track 4) were once again subjected to SDS-PAGE electrophoresis and Western blotting. The resultant nitrocellulose filter was screened with (i) the anti-65KD M. leprae monoclonal antibody ML30 and then (ii) alkaline phosphatase conjugated secondary antibodies.

Following development of the filter in the phosphatase colour solution (Photograph 7), antigenic bands of c.65KD were visualised in tracks 1, 2 and 3. This result shows that (i) the anti-65KD M. leprae monoclonal antibody ML30, recognises an epitope within the 65KD M. leprae protein expressed by clone Y3178 (track 3), as expected and (ii) the 65KD M. bovis BCG protein expressed by pBR322/M. bovis BCG clone A (track 1) and clone pRIB1000 (track 2) (both clones are expressing the same 65KD M. bovis BCG antigen; section 10.2.1) also contains the epitope recognised by ML30. The 64KD M. fortuitum protein (expressed by clone pAL15) was not detected on the filter (track 4), implying that this protein does not contain the epitope recognised by ML30. In addition to the 65KD antigenic proteins, a number of other antigenic bands reacted strongly in tracks 1, 2 and 3 (and these bands were similarly detected in track 4), these are E. coli bands and these reactions imply a high degree of cross-reactivity between the M. leprae monoclonal antibody ML30 and a number of E. coli proteins.
The results of the experiments described in sections 10.2.1 and 10.2.2 confirm a high degree of cross-reactivity between the \textit{M. bovis} BCG 65KD antigen (expressed by pER322/\textit{M. bovis} BCG clone A and clone RIB1000) and the \textit{M. leprae} 65KD antigen (expressed by clone Y3178). Moreover, no cross-reactivity was observed between these two 65KD antigens and the 64KD \textit{M. fortuitum} protein expressed by clone pAL15, thus this \textit{M. fortuitum} protein is not related to the 65KD \textit{M. bovis} BCG and \textit{M. leprae} proteins.
Photograph 7: Recombinant Mycobacterial Proteins Screened with Monoclonal Antibody ML30

Track number

1 = pBR322/M. bovis BCG clone A
2 = clone pRIB1000
3 = clone Y3178
4 = clone pAL15

The Filter was screened with (i) a 1:500 dilution of the anti-65KD M. leprae monoclonal antibody ML30 and (ii) a 1:1000 dilution of alkaline phosphatase conjugated donkey anti-mouse IgG.
10.3 In Situ Screening of the gtII/M. bovis BCG Library

Four aliquots (1.6ml) of E.coli Y1090 cells were each mixed with c.5 x 10⁴ pfu from the gtII/M.bovis BCG library and the resultant infection mixtures were plated out onto L-Agar plates, which were then incubated at 42°C (section 6.10.1). Following 3 hours incubation at 42°C, each plate was overlaid with an IPTG-soaked nitrocellulose filter and the plates + filters were then incubated at 37°C. During the 3 hour incubation period, expressed proteins were immobilised on the nitrocellulose filters. Two screening protocols were subsequently employed to detect clones expressing M.bovis BCG antigens.

One of the nitrocellulose filters was screened with (i) E.coli absorbed rabbit anti-M.bovis BCG antiserum; (ii) biotin conjugated secondary antibodies and (iii) a streptavidin-biotinylated peroxidase solution, as described in section 6.10.1. Following incubation of the filter in the peroxidase colour development solution, two positive signals were detected. Re-aligning the filter with the L-Agar plate identified the plaques responsible for the positive signals (i.e. gtII/M.bovis BCG clones 531-6 and 532-9; the two clones expressing the M.bovis BCG antigenic proteins).

The remaining three nitrocellulose filters were each screened with (i) E.coli absorbed rabbit anti-M.bovis BCG antiserum and (ii) alkaline phosphatase conjugated secondary antibodies, as described in section 6.10.1. Incubation of these three filters in the phosphatase colour development solution resulted in the detection of five positive
signals and re-aligning the filters with the corresponding L-Agar plates identified the plaques responsible for these positive signals (i.e. \textit{gtII/M. bovis} BCG clones 257-1, 257-2, 306-1, 306-2 and 306-3).

It was not possible to pick the seven plaques (described above) from their respective plates individually (the plaque density was very high on all four plates), thus small areas, surrounding and including the seven positive plaques, were scraped from the plates and the material was dispersed in 50\(\mu\)l volumes of SM buffer, producing amplified phage stocks of the seven clones. To plaque purify these seven stocks, a dilution of each was plated out onto an L-Agar plate and the resultant plaques (on each of the seven plates) were re-screened for antigen-expression as described in section 6.11. A single positive plaque was subsequently selected from each of the seven plates and transferred, with the aid of a toothpick, into a 50\(\mu\)l volume of SM buffer to produce a plaque-purified phage stock of each of the seven \textit{M. bovis} BCG antigen-expressing clones.

At this time, two of my colleagues (M.E. Collins and S. Wall) showed that positive signals that they had previously obtained, following their screening of a mycobacterial gene library with the streptavidin system, had been obtained independently of the binding of primary antibody and were thus false positives. Consequently, the experiments described below, were carried out to determine whether the two clones (i.e. \textit{gtII/M. bovis} BCG clones 531-6 and 532-9), obtained following my screening of the \textit{gtII/M. bovis} BCG library with the streptavidin system, were also false positives.
Aliquots of the plaque-purified phage stocks of *gtII/M. bovis* BCG clones 531-6 and 532-9 were plated out onto two L-Agar plates. After c.3 hours incubation at 42°C, the plates were overlaid with IPTG-soaked nitrocellulose filters and the plates + filters were then incubated at 37°C for c.3 hours. Following their removal from the L-Agar plates, the two filters were then cut into two equal portions (i.e. A- and B-fragments) and these were then screened as follows: (1) the A-fragments were screened with *E. coli* absorbed rabbit anti-*M. bovis* BCG antisera; biotin conjugated secondary antibodies and then the streptavidin-biotinylated horseradish peroxidase solution (as described in section 6.10.1) and (2) the B-fragments were screened with the streptavidin-biotinylated horseradish peroxidase solution only. Both sets of filters were then incubated in the peroxidase colour development solution: positive signals to the *M. bovis* BCG protein expressed by *gtII/M. bovis* BCG clone 532-9 were only detected on its A-fragment filter and not on its B-fragment filter (Photograph 8); positive signals to the *M. bovis* BCG protein expressed by *gtII/M. bovis* BCG clone 531-6 were detected on both its A- and B-fragment filters (Photograph 9).

The detection of positive signals on the B-fragment filter of *gtII/M. bovis* BCG clone 531-6 was unexpected, as this fragment had been screened in the absence of primary antibody. Consequently, these signals are false positives, detected solely as a result of the ability of the *M. bovis* BCG protein expressed by *gtII/M. bovis* BCG clone 531-6 to bind the streptavidin-biotinylated horseradish peroxidase complex. This result implies that this clone is expressing a biotinylated *M. bovis* BCG protein (see section 11.2 for further details of this biotinylated
protein and sections 13.3 and 14.1 for details of the identification and characterisation of other biotinylated mycobacterial proteins). The results with gtII/M. bovis BCG clone 532-9 were as expected: positive signals when screened with primary antibody; no signals when screened in the absence of primary antibody and imply that this clone is expressing an antigenic M. bovis BCG protein (see sections 11.3 and 14.3 for further characterisation of this antigenic protein).

Photograph 8: A Plaque-Purified Phage Stock of gtII/M. bovis BCG clone 532-9 Screened With and Without Primary Antibody

A-fragment: screened with (i) a 1:500 dilution of E. coli absorbed rabbit anti-M. bovis BCG antiserum; (ii) a 1:2000 dilution of biotin conjugated goat anti-rabbit IgG and (iii) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution.

B-fragment: screened with (i) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution (i.e. no antibody treatment).
Photograph 9: A Plaque-Purified Phage Stock of ªII/M. bovis BCG clone 531-6 Screened With and Without Primary Antibody

**A-fragment**: screened with (i) a 1:500 dilution of *E. coli* absorbed rabbit anti-*M. bovis* BCG antiserum; (ii) a 1:2000 dilution of biotin conjugated goat anti-rabbit IgG and (iii) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution.

**B-fragment**: screened with (i) a 1:500 dilution of a streptavidin-biotinylated horseradish peroxidase complex solution (i.e. no antibody treatment).
In Situ Screening of the EMBL4/M. bovis BCG Library

The EMBL4 cloning vector can accommodate larger foreign DNA insert fragments (9-23Kb) than either (i) plasmid vectors or (ii) the phage lambda vector gtII. Thus, by cloning M. bovis BCG DNA into this vector, I hoped to increase the likelihood of producing recombinant clones containing gene clusters, coding for entire biosynthetic pathways.

Two aliquots (1.6ml) of E. coli NM539 cells were each mixed with c.5 x 10⁴ pfu from the EMBL4/M. bovis BCG library and the resultant infection mixtures were plated out onto L-Agar plates, then incubated at 37°C for c.7 hours, as described in section 6.10.2. The plates were then overlaid with nitrocellulose filters and the plates + filters were incubated at 37°C for a further 3 hours.

Following their removal from the L-Agar plates, the nitrocellulose filters were screened with (i) a 1:500 dilution of E. coli absorbed rabbit anti-M. bovis BCG antiserum and then (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG. Incubation of the filters in the phosphatase colour development solution followed but no positive signals were detected on either filter.

As the phage lambda vector EMBL4 is not an expression vector, the expression of M. bovis BCG genes cloned into this vector is dependent upon recognition of their cloned M. bovis BCG promoter sequences by E. coli RNA polymerase. The failure to detect M. bovis BCG gene
expression appears to confirm the findings made earlier (section 8.2), i.e. the majority of *M. bovis* BCG promoters are only weakly active in *E. coli*.

Thole et al. (1985) screened c.5 x 10^3 EMBL3/*M. bovis* BCG clones by this same in situ screening technique and similarly failed to detect clones expressing *M. bovis* BCG antigenic proteins. However, by screening EMBL3/*M. bovis* BCG clones by Western blotting, these authors were able to detect clones expressing *M. bovis* BCG antigens. As I had already obtained a number of clones (from other libraries i.e. pBR322/*M. bovis* BCG and gtII/*M. bovis* BCG) expressing *M. bovis* BCG antigenic proteins, I decided not to continue screening the EMBL4/*M. bovis* BCG library.
Further characterisation of the antigenic \textit{M. bovis} BCG proteins expressed by \textit{gtII/M. bovis} BCG clones 532-9, 257-1, 257-2, 306-1, 306-2 and 306-3 and the biotinylated \textit{M. bovis} BCG protein expressed by \textit{gtII/M. bovis} BCG clone 531-6, required the preparation of lysogens for each of the recombinant clones. These lysogens then enabled sufficient quantities of the expressed proteins to be prepared, to allow their subsequent analysis by SDS-PAGE electrophoresis and Western blotting.

11.1 The Production of Lysogens and Crude Protein Lysates

Aliquots of \textit{E. coli} Y1089 cells were lysogenised with phage from each of the seven recombinant clones, in accordance with the protocol described in section 6.12. The resultant cells + phage mixtures were then plated out onto L-Agar plates, which were then incubated at 30°C. Following overnight incubation at 30°C, numerous colonies were detected on each of the seven L-Agar plates.

One hundred colonies were selected from each of the seven plates and these were then stabbed onto two sets of L-Agar plates, with the aid of toothpicks. One set of plates was subsequently incubated at 30°C overnight and the second set of plates was incubated at 42°C overnight. Table 25 contains the results of the seven "lysogen experiments", and those colonies growing at 30°C but not at 42°C, were considered to be lysogenic.
The observed variability in the percentage of lysogens obtained, was a consequence of the ratio of phage to cells in the lysogen infection mixtures. Originally infection mixtures were prepared with a phage to cell ratio of 5:1 (i.e. this ratio was used for the production of lysogens to gtII/M.bovis BCG clones 531-6, 532-9, 306-1, 306-2 and 306-3) however, due to the low frequency of lysogens obtained, i.e. 1, 3, 8, 8 and 4 lysogens respectively, the phage to cell ratio was subsequently increased to 100:1 for the production of lysogens to the gtII/M.bovis BCG clones 257-1 and 257-2.

Seven lysogens were subsequently selected, one for each of the seven recombinant clones and their status as lysogens was confirmed by their growth at 30°C but not at 42°C. Crude protein lysate samples were then prepared, in accordance with the protocol described in section 6.12.
**Table 25: Numbers of Lysogens**

<table>
<thead>
<tr>
<th>Colonies Tested</th>
<th>Numbers Growing at 30°C</th>
<th>Numbers Growing at 42°C</th>
<th>Numbers of Lysogens</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 x gtII/M. bovis BCG clone 531-6 colonies</td>
<td>100/100</td>
<td>99/100</td>
<td>1</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 532-9 colonies</td>
<td>100/100</td>
<td>97/100</td>
<td>3</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 306-1 colonies</td>
<td>100/100</td>
<td>92/100</td>
<td>8</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 306-2 colonies</td>
<td>100/100</td>
<td>92/100</td>
<td>8</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 306-3 colonies</td>
<td>100/100</td>
<td>96/100</td>
<td>4</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 257-1 colonies</td>
<td>100/100</td>
<td>4/100</td>
<td>96</td>
</tr>
<tr>
<td>100 x gtII/M. bovis BCG clone 257-2 colonies</td>
<td>100/100</td>
<td>15/100</td>
<td>85</td>
</tr>
</tbody>
</table>
11.2 Western Blot Analysis of the Biotinylated M. bovis BCG Protein Expressed by gtII/M. bovis BCG Clone 531-6

Following its preparation (see section 6.12), crude protein extract of gtII/M. bovis BCG clone 531-6 was subjected to SDS-PAGE electrophoresis, together with a soluble extract of M. bovis BCG. Following electrophoresis the SDS-PAGE gel was Western blotted (section 6.13.2); the resultant nitrocellulose filter was then incubated in a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution and the filter was then incubated in the peroxidase colour development solution.

Photograph 10 shows a single band in track 1 (the gtII/M. bovis BCG clone 531-6 extract), corresponding to a protein of c.85KD. In track 2 (the M. bovis BCG extract sample), three bands were observed to react with the streptavidin-biotinylated horseradish peroxidase complex solution and the sizes of these biotinylated M. bovis BCG proteins were estimated to be 120KD, 85KD and 65KD. From this photograph it is clear that one of the biotinylated proteins in the M. bovis BCG extract sample (the 85KD protein band) is identical in size to the biotinylated protein expressed by gtII/M. bovis BCG clone 531-6 and therefore is presumably the same protein. The identification of a similar sized biotinylated protein within the M. bovis BCG extract and the fact that the minimum size for a fusion protein (with beta-galactosidase) is 114KD, shows that the protein expressed by gtII/M. bovis BCG clone 531-6 is not a fusion and raises the possibility that this protein is being expressed from its own promoter.
In an attempt to determine whether this 85KD biotinylated *M. bovis* BCG protein was being expressed from its own promoter, two further crude protein extracts of gtII/*M. bovis* BCG clone 531-6 were made. During preparation however, only one of the two samples was induced with 10mM IPTG (the second sample was prepared in the absence of IPTG induction). Aliquots of these two, newly prepared, crude protein extracts were then subjected to SDS-PAGE electrophoresis and Western blotting, as described in sections 6.13.1 and 6.13.2.

The nitrocellulose filter was incubated in a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution and then developed in the peroxidase colour solution. Photograph 11 shows that expression of the c.85KD biotinylated protein was greater in the presence of IPTG (track 1) than in its absence (track 2) and this result implies that this protein is partly inducible with IPTG. Moreover, this result further suggests that the *M. bovis* BCG promoter for this gene is either absent or inactive (in *E. coli*) and that the gene is co-transcribed with the beta-galactosidase gene of the vector. The absence of a fusion protein suggests that translation of this c.85KD protein is initiated at an independant ribosome binding site.
Photograph 10: Analysis of the Biotinylated M.bovis BCG Protein
Expressed by gtII/M.bovis BCG clone 531-6

Track number
1 = gtII/M.bovis BCG clone 531-6
2 = M.bovis BCG

The filter was screened with a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution and then developed in the peroxidase colour development solution (i.e. no antibody treatment).
Expression of the 85KD Biotinylated M. bovis Protein by gtII/M. bovis BCG clone 531-6 in the Presence and the Absence of IPTG

Track number

1 = gtII/M. bovis BCG clone 531-6 (+IPTG)
2 = gtII/M. bovis BCG clone 531-6 (-IPTG)
3 = M. bovis BCG

The filter was screened with a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution and then developed in the peroxidase colour development solution (i.e. no antibody treatment).
Following the detection of three biotinylated *M. bovis* BCG proteins (Photographs 10 and 11), soluble extracts of (i) *M. phlei*; (ii) *M. vaccae*; (iii) *M. smegmatis* and (iv) *M."lufu"*, were prepared, in order to allow the determination of the numbers and sizes of biotinylated proteins expressed by each of these mycobacterial species. Extracts of the four species (together with *M. bovis* BCG) were subsequently subjected to SDS-PAGE electrophoresis and Western blotting. Following blotting the nitrocellulose filter was incubated in a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution, then developed in the peroxidase colour solution.

Each of the five mycobacterial species tested, expressed three biotinylated molecules, i.e. c.65KD, c.85KD and c.120KD in size (data not shown) and in each case the c.65KD molecule was the major biotinylated molecule expressed.
Western Blot Analysis of the Antigenic M. bovis BCG Proteins
Expressed by Six gtII/M. bovis BCG Clones

Crude protein extracts were prepared from lysogens of the gtII/M. bovis BCG clones 532-9, 257-1, 257-2, 306-1, 306-2 and 306-3 (as described in section 6.12) and aliquots of each were then subjected to SDS-PAGE electrophoresis and Western blotting. The resultant nitrocellulose filter was screened with (i) *E. coli* absorbed rabbit anti-M. bovis BCG antiserum and (ii) alkaline phosphatase conjugated secondary antibodies and then developed in the phosphatase colour solution (section 6.13.3).

Photograph 12 shows the antigenic proteins (marked with ---) expressed by each of the six gtII/M. bovis BCG clones: the antigenic protein expressed by gtII/M. bovis BCG clone 257-2 (track 2) was estimated to be 80KD; the antigenic proteins expressed by each of the five other gtII/M. bovis BCG clones (tracks 1, 4, 5, 6 and 8) were all estimated to be larger than 116KD. As the minimum size for an expressed fusion protein is 114KD, the results in Photograph 12 show that the protein expressed by gtII/M. bovis BCG clone 257-2 is not a fusion (which raises the possibility that this protein is being expressed from its own promoter) but show that the antigenic proteins expressed by all of the other five gtII/M. bovis BCG clones may be fusions. Apart from the antigenic *M. bovis* BCG protein bands, a number of other bands were also observed in tracks 1, 2, 4, 5, 6 and 8 however, comparison with the bands in tracks 3 and 7 (both of which contain a non-recombinant *E. coli* lysogen) suggests that these background bands are *E. coli* and phage
lambda gtII proteins, which are able to cross-react with antibodies present in the anti-\textit{M. bovis} BCG antisera.

In order to determine whether the high molecular weight antigenic proteins expressed by gtII/\textit{M. bovis} BCG clones 532-9, 257-1, 306-1, 306-2 and 306-3 were fusion proteins (i.e., expressed in conjunction with beta-galactosidase) crude protein extracts of these five clones were subjected to SDS-PAGE electrophoresis and then Western blotting. The nitrocellulose filter was then screened with (i) a 1:1000 dilution of an anti-beta-galactosidase monoclonal antibody and (ii) alkaline phosphatase conjugated secondary antibodies. Development in the phosphatase colour solution produced banding patterns on the filter, similar to those shown in Photograph 12. Thus, the high molecular weight antigenic proteins expressed by each of the gtII/\textit{M. bovis} BCG clones had each reacted with anti-beta-galactosidase antibodies (data not shown), confirming that each of these expressed antigens \textit{M. bovis} BCG proteins is a fusion protein.
Western Blot Analysis of the Antigenic *M. bovis* BCG Proteins Expressed by Six *gtII/M. bovis* BCG Clones

**Track number**

1 = *gtII/M. bovis* BCG clone 257-1  
2 = *gtII/M. bovis* BCG clone 257-2  
3 = *E. coli* Y1089  
4 = *gtII/M. bovis* BCG clone 532-9  
5 = *gtII/M. bovis* BCG clone 306-1  
6 = *gtII/M. bovis* BCG clone 306-2  
7 = *E. coli* Y1089  
8 = *gtII/M. bovis* BCG clone 306-3

The filter was screened with (i) a 1:500 dilution of *E. coli* absorbed rabbit anti-*M. bovis* BCG antiserum and (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG.
Further characterisation of the antigenic fusion proteins expressed by gtl1/M. bovis BCG clones 532-9, 257-1, 306-1, 306-2 and 306-3 was attempted with the aid of three monoclonal antibodies (each of which was reactive with M. bovis BCG): TB78, raised against a 65KD protein; TB71, raised against a 38KD protein and TB68 raised against a 14KD protein (Coates et al., 1981; Bothamley et al., 1987).

Crude protein extracts of the five gtl1/M. bovis BCG clones listed above, together with a soluble extract of M. bovis BCG were loaded onto three SDS-PAGE gels. Following electrophoresis the gels were Western blotted and the resultant nitrocellulose filters were incubated as follows: Filter 1 with (i) a 1:500 dilution of TB78 and (ii) alkaline phosphatase conjugated secondary antibodies; Filter 2 with (i) a 1:500 dilution of TB71 and (ii) alkaline phosphatase conjugated secondary antibodies and Filter 3 with (i) a 1:500 dilution of TB68 and (ii) alkaline phosphatase conjugated secondary antibodies. The filters were then developed in the phosphatase colour solution.

As Table 26 shows, none of the expressed antigenic fusion proteins reacted with any of the three monoclonal antibodies (the reactivity of the monoclonal antibodies was verified by the positive signals obtained with the M. bovis BCG extract). These results may imply that these clones express M. bovis BCG proteins, other than those recognised by the monoclonal antibodies TB78, TB71 and TB68. However it is also possible that the negative results obtained may have been
achieved because the specific epitopes recognised by these three monoclonal antibodies were not present within the expressed fusion proteins or they may have been destroyed by the folding of the fusion proteins. Consequently to characterise the antigenic fusion proteins expressed by gtII/M.bovis BCG clones 532-9, 257-1, 306-1, 306-2 and 306-3 it will be necessary to use monoclonal antibodies recognising other epitopes of the 65KD, 38KD and 14KD proteins, together with those monoclonal antibodies recognising other mycobacterial proteins.

Table 26: Screening the Expressed Antigenic M.bovis BCG Proteins with Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reaction with Monoclonal Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB78</td>
</tr>
<tr>
<td>gtII/M.bovis BCG clone 532-9</td>
<td>None</td>
</tr>
<tr>
<td>gtII/M.bovis BCG clone 257-1</td>
<td>None</td>
</tr>
<tr>
<td>gtII/M.bovis BCG clone 306-1</td>
<td>None</td>
</tr>
<tr>
<td>gtII/M.bovis BCG clone 306-2</td>
<td>None</td>
</tr>
<tr>
<td>gtII/M.bovis BCG clone 306-3</td>
<td>None</td>
</tr>
<tr>
<td>M.bovis BCG</td>
<td>Yes</td>
</tr>
</tbody>
</table>
11.5 Size Determination of the Cloned *M. bovis* BCG DNA Inserts

Amplified phage stocks of gtII/*M. bovis* BCG clones 531-6, 532-9, 257-1, 257-2, 306-1, 306-2 and 306-3 were prepared and then recombinant phage DNA was extracted from each, in accordance with the procedure described in section 6.4.2. As the recombinant clones had been prepared through the ligation of *M. bovis* BCG DNA fragments into the EcoRI cloning site of gtII, the *M. bovis* BCG DNA inserts were removed by digestion of the recombinant phage DNA samples with EcoRI, as follows:

**Typical EcoRI digestion**

1μg recombinant phage DNA + 1μl 10X High buffer + 3μl distilled water + 120 units EcoRI

Following digestion at 37°C for 1 hour, the digests were analysed by agarose gel electrophoresis (Photographs 13, 14 and 15) and Table 27 below, lists the sizes of the *M. bovis* BCG DNA inserts of each of the seven recombinant clones.
<table>
<thead>
<tr>
<th>Recombinant Clone</th>
<th>Size of Cloned Insert (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gtII/M. bovis BCG clone 531-6</td>
<td>6.0</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 532-9</td>
<td>3.5</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 257-1</td>
<td>3.0</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 257-2</td>
<td>3.0</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 306-1</td>
<td>4.3</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 306-2</td>
<td>2.1</td>
</tr>
<tr>
<td>gtII/M. bovis BCG clone 306-3</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Photograph 13: Restriction Enzyme Analysis of gtII/M. bovis BCG
Clones 531-6, 257-1, 257-2 and 306-1

Track number

1 = EcoRI digest of gtII/M. bovis BCG clone 257-1 DNA
2 = EcoRI digest of gtII/M. bovis BCG clone 257-2 DNA
3 = HindIII digest of Lambda DNA
4 = EcoRI digest of gtII/M. bovis BCG clone 531-6 DNA
5 = EcoRI digest of gtII/M. bovis BCG clone 306-1 DNA

Photograph 14: Restriction Enzyme Analysis of gtII/M. bovis BCG
Clones 306-2 and 306-3

Track number

1 = EcoRI digest of gtII/M. bovis BCG clone 306-2
2 = EcoRI digest of gtII/M. bovis BCG clone 306-3
5 = HindIII digest of Lambda DNA

Photograph 15: Restriction Enzyme Analysis of gtII/M. bovis BCG
Clone 532-9

Track number

1 = HindIII digest of Lambda DNA
2 = EcoRI digest of gtII/M. bovis BCG clone 532-9

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Photograph 13: Restriction Enzyme Analysis of gtII/M. bovis BCG Clones 531-6, 257-1, 257-2 and 306-1

Photograph 14: Restriction Enzyme Analysis of gtII/M. bovis BCG Clones 306-2 and 306-3
Photograph 15: Restriction Enzyme Analysis of \textit{gtII/M. bovis BCG Clone 532-9}
Section 12 Screening M. leprae DNA Gene Libraries with Monoclonal Antibodies

Using the mouse anti-M. leprae monoclonal antibodies ML10, raised against a 12KD protein; ML30, raised against a 65KD protein and ML38, raised against a 36KD protein (Ivanyi et al., 1983), aliquots of the pBR322/M. leprae, pUC13/M. leprae and gtII/M. leprae libraries were screened to identify clones expressing antigenic M. leprae proteins.

12.1 Screening pBR322/M. leprae and pUC13/M. leprae Clones

With the aid of toothpicks, L-Agar plates (containing 50μg/ml ampicillin) were inoculated with 768 clones from the pBR322/M. leprae library and 1632 clones from the pUC13/M. leprae library and the plates were subsequently incubated at 37°C. Following overnight incubation, each plate was overlaid with a nitrocellulose filter and the plates + filters were re-incubated at 37°C for a further 5 minutes. When the filters were removed from the plates, cells remained bound to the filters and these cells were lysed on the filters, in accordance with the procedure described in section 6.9.

The nitrocellulose filters were then incubated in a solution containing each of the three monoclonal antibodies (i.e. ML10 + ML30 + ML38) and bound primary antibody was in turn detected with horseradish peroxidase conjugated secondary antibodies (section 6.9). Following incubation of the filters in the peroxidase colour development solution, colour reactions were observed with all of the clones (probably due to
endogenous peroxidase activity reacting with the peroxidase colour development solution), nevertheless twelve clones (i.e. six pBR322/\textit{M. leprae} and six pUC13/\textit{M. leprae}) were selected for testing by Western blotting.

Overnight cultures of the twelve clones were initially prepared, 1ml aliquots of each culture were harvested and the pellets were then resuspended in SDS-PAGE loading buffer. An aliquot of each sample was then subjected to SDS-PAGE electrophoresis and then Western blotting. Following blotting, the nitrocellulose filter was incubated in (i) the primary antibody solution (consisting of ML10, ML30 and ML38) and then (ii) incubated with horseradish peroxidase conjugated secondary antibodies, as described in section 6.9. Following development of the filter in the peroxidase colour solution, none of the twelve clones showed a clear antigenic \textit{M. leprae} protein band, reactive with any of the three monoclonal antibodies (data not shown), only \textit{E. coli} protein bands reacted positively (the monoclonal antibody ML30 is cross-reactive with an \textit{E. coli} antigen).

Two reasons have been suggested for the failure to detect clones expressing the 12KD, 36KD and 65KD antigenic \textit{M. leprae} proteins: (1) too few clones were screened and (2) it is possible that the \textit{M. leprae} promoters for these genes are not active in \textit{E. coli}, thus the antigenic proteins were not detected because they were not expressed. (Point 2 cannot be used to explain the failure to detect clones expressing the 65KD protein, as R. Young and his colleagues have shown that the 65KD \textit{M. leprae} protein is expressed from its own promoter in \textit{E. coli}).
12.2 Screening gtII/M.leprae Clones

An aliquot (1.6ml) of E.coli Y1090 cells was mixed with c.2 x $10^2$ pfu from the gtII/M.leprae library and the resultant infection mixture was plated out onto an L-Agar plate. Following incubation at 42°C for 3 hours, the plate was overlaid with an IPTG-soaked nitrocellulose filter and the plate + filter was then incubated at 37°C.

Following 3 hours incubation at 37°C, the filter was removed from the L-Agar plate and incubated with (i) monoclonal antibody ML30 and then (ii) alkaline phosphatase conjugated secondary antibodies, as described in section 6.10.1. The filter was subsequently incubated in the phosphatase colour development solution and two positive signals were detected. Re-aligning the filter with the L-Agar plate, allowed the plaques responsible for the positive signals to be identified (i.e. gtII/M.leprae clones ML-301 and ML-302).

Due to the high plaque density on the plate, it was impossible to pick the positive plaques from the plate discretely, thus a small number of plaques, surrounding and including the positive plaques, were scraped from the plate and this material was dispersed in 50μl volumes of SM buffer. Plaque-purified phage stocks of these two positive clones were subsequently prepared, in accordance with the procedure described in section 6.11.

When this stage was reached, results were reported by Young et al. (1985b), that described the isolation of a gtII clone expressing a
65KD \textit{M. leprae} antigen (the \textit{M. leprae} antigen recognised by ML30). Thus no further attempt was made to characterise gtII/\textit{M. leprae} clones ML-301 and ML-302. As my source of \textit{M. leprae} was different from R. Young's it may be interesting to compare the DNA sequences of the respective clones, however, as three groups have recently published the DNA sequence of the 65KD antigen from \textit{M. leprae}, \textit{M. tuberculosis} and \textit{M. bovis} BCG (Mehra et al., 1986; Shinnick, 1987 and Thole et al., 1987) and shown this gene to be highly conserved, it is unlikely that intra-species variation of this gene will be very great.
Section 13  Screening _M_.leprae DNA Gene Libraries with Sera from Leprosy Patients

To date, with the aid of a number of mouse anti-_M_.leprae monoclonal antibodies, clones expressing five antigenic _M_.leprae proteins (i.e. 12KD, 18KD, 28KD, 36KD and 65KD _M_.leprae proteins) have been identified and isolated from a gt11/ _M_.leprae library (Young et al., 1985b). Other groups however, using sera from human leprosy patients, have identified a number of different _M_.leprae antigens, not recognised by the available mouse monoclonal antibodies. In particular, Chakrabarty et al. (1982) and Klatser et al. (1984) identified 22KD, 33KD, 41KD and 86KD _M_.leprae antigens. Consequently, by screening my _M_.leprae gene libraries with sera from leprosy patients (kindly provided by Dr J. L. Stanford), I hoped to identify recombinant clones expressing _M_.leprae antigens, other than those identified by Young et al. (1985b).

13.1 Screening pBR322/_M_.leprae Clones

Diluted aliquots of the amplified pBR322/_M_.leprae library (section 9.2) were prepared and plated out onto six L-Agar plates, which were then incubated at 37°C. Following overnight incubation at 37°C, each of the plates (containing c.500 colonies) was overlaid with a nitrocellulose filter and the plates + filters were incubated at 37°C for a further 5 minutes. Following their removal from the plates, the filters were treated as described in section 6.9 (to lyse the cells bound to the filters). The filters were then incubated with (i) _E_.coli absorbed serum from a lepromatous leprosy patient and (ii) alkaline
phosphatase conjugated secondary antibodies, as described in section 6.9. The filters were finally incubated in the phosphatase colour development solution.

Despite screening c.3000 pBR322/M. leprae clones, I failed to detect any clones expressing antigenic M. leprae proteins. Again, the low activity of the majority of M. leprae promoters in E. coli and the low number of clones screened, may account for this failure to detect antigen-expression clones.

13.2 Screening gtII/M. leprae Clones with Phosphatase-Conjugated Secondary Antibodies

Three aliquots of E. coli Y1090 cells were each mixed with c.5 x 10^4 pfu from the gtII/M. leprae library and the resultant infection mixtures were then plated out onto L-Agar plates, as described in section 6.10.1. Following 3 hours incubation at 42°C, each of the plates was overlaid with an IPTG-soaked nitrocellulose filter and plates + filters were then incubated at 37°C. After 3 hours incubation, the filters were removed from the L-Agar plates and screened as described in section 6.10.1: Filter 1 was incubated with E. coli absorbed serum from a lepromatous leprosy patient (LL serum No. 6); Filter 2, with E. coli absorbed serum from a tuberculoid leprosy patient (TT serum No. 29) and Filter 3, with E. coli absorbed serum from a close-contact of leprosy patients (CC serum No. 516). Bound primary antibody was subsequently detected with alkaline phosphatase conjugated secondary antibodies.
The filters were then incubated in the phosphatase colour development solution, however no positive signals were detected on any of the filters. It was however anticipated that (i) the low numbers of recombinant clones screened with each serum sample (c.2 x 10^4 per filter) and (ii) the insensitivity of the in situ phosphatase-linked immunoassay accounted for these results.

13.3 Screening gtII/M.leprae Clones with Biotin Conjugated Secondary Antibodies

Four aliquots (1.6ml) of E.coli Y1090 cells were infected with c.5 x 10^4 pfu from the gtII/M.leprae library and the cells + phage mixtures were plated out on L-Agar plates (section 6.10.1). Following incubation at 42°C, the plates were overlaid with IPTG-soaked nitrocellulose filters and incubated at 37°C. Each of the filters was then screened with (i) E.coli absorbed serum from a tuberculoid leprosy patient (TT serum No. 29); (ii) biotin conjugated secondary antibodies and then (iii) with the streptavidin-biotinylated horseradish peroxidase complex solution, as described in section 6.10.1. Following development of the filters in the peroxidase colour solution, three positive signals were detected. Re-aligning the filters with the corresponding plates, allowed the plaques responsible for the positive signals to be identified (i.e. gtII/M.leprae clones SAB-2, SAB-7 and SAB-8). The positive plaques were removed from the plates and plaque-purified phage stocks of each, were subsequently prepared (section 6.11).
Previously (section 10.3), I described the detection of a false-positive gtII/M.bovis BCG clone (obtained following the screening of the gtII/M.bovis BCG library with the streptavidin system). Therefore, to determine whether these three gtII/M.leprae clones were false positives (i.e. were the positive signals obtained independently of the binding of the primary antibody?) the following test was carried out. An aliquot of each of the three plaque-purified phage stocks was plated out onto L-Agar plates and these were then incubated at 42°C. After 3 hours incubation, the plates were overlaid with IPTG-soaked nitrocellulose filters and then incubated at 37°C. Following their removal from the plates, each of the filters was cut into two equal portions (i.e. A- and B- fragments) and screened as follows: (1) the A-fragments were incubated with E.coli absorbed TT serum Ho. 29; biotin conjugated secondary antibodies and then the streptavidin-biotinylated horseradish peroxidase solution and (2) the B-fragments were incubated with the streptavidin-biotinylated horseradish peroxidase solution only. Following incubation of both sets of filters (i.e. the A- and B-fragments) in the peroxidase colour solution, positive signals were detected on all of the A- and all of the B-fragments, i.e. positive signals were detected, even in the absence of primary antibody, for each of the three clones. Thus, like gtII/M.bovis BCG clone 531-6 described earlier (section 10.3), the gtII/M.leprae clones SAB-2, SAB-7 and SAB-8 are each expressing a biotinylated molecule.

Amplified phage stocks of these three gtII/M.leprae clones were prepared and recombinant phage DNA was subsequently extracted from each stock, as described in section 6.4.2. To determine the sizes of the
cloned \textit{M. leprae} DNA inserts, an aliquot of each recombinant phage DNA stock was digested with EcoRI and the three digests were then analysed by agarose gel electrophoresis. Identical digestion patterns were observed for the three clones, i.e. each clone had two EcoRI insert fragments of 4.3Kb and 1.0Kb (Photograph 16). This result suggested that the three \textit{gtlI/M. leprae} clones (SAB-2, SAB-7 and SAB-8) were all re-isolations of the same clone (containing an internal EcoRI site), therefore further work, to characterise the biotinylated \textit{M. leprae} protein expressed by these clones was restricted to \textit{gtlI/M. leprae} clone SAB-2.

Following the procedure described in section 6.12, crude protein extract was prepared from a \textit{gtlI/M. leprae} clone SAB-2 lysogen and subjected to SDS-PAGE electrophoresis, together with an extract of \textit{gtlI/M. bovis} BCG clone 531-6 (expressing a biotinylated \textit{M. bovis} BCG protein) and \textit{M. bovis} BCG. Following electrophoresis the SDS-PAGE gel was Western blotted and the nitrocellulose filter was incubated with the streptavidin-biotinylated horseradish peroxidase complex solution. Incubation of the filter in the peroxidase colour solution subsequently revealed (Photograph 17) that \textit{gtlI/M. leprae} clone SAB-2 (track 1) was expressing a similar sized biotinylated protein, to that expressed by \textit{gtlI/M. bovis} BCG clone 531-6 (tracks 2). Thus, \textit{gtlI/M. leprae} clone SAB-2 was expressing an c.85KD biotinylated \textit{M. leprae} protein.
Photograph 16: Restriction Enzyme Analysis of Three gtII/M.leprae Clones

Track number:
1 = HindIII digest of Lambda DNA
2 = HaeIII digest of φX174 DNA
4 = EcoRI digest of gtII/M.leprae clone SAB-2
5 = EcoRI digest of gtII/M.leprae clone SAB-7
6 = EcoRI digest of gtII/M.leprae clone SAB-8
Photograph 17: Analysis of the Biotinylated \textit{M. leprae} Protein
Expressed by \textit{gt}II/\textit{M. leprae} Clone SAB-2

\textbf{Track number}

1 = \textit{gt}II/\textit{M. leprae} clone SAB-2
2 = \textit{gt}II/\textit{M. bovis} clone 531-6
3 = \textit{M. bovis} BCG

The filter was screened with a 1:500 dilution of the streptavidin-
biotinylated horseradish peroxidase complex solution and then developed
in the peroxidase colour development solution (i.e. no antibody
treatment).
Section 14 Analysis of Expression Positive gtII/M. leprae Clones with Sera from Leprosy Patients

Having screened the gtII/M. leprae library with sera from leprosy patients but failed to detect clones expressing antigenic M. leprae proteins, I attempted to test the methodology (in situ enzyme-linked immunoassay) with known expression positive clones. The gtII/M. leprae clones Y3164 (expressing a 28KD M. leprae protein), Y3178 (65KD M. leprae protein), Y3179 (18KD M. leprae protein), Y3180 (36KD M. leprae protein) and Y3184 (12KD M. leprae protein) were subsequently obtained from Dr. R. A. Young (as plaque-purified phage stocks) and aliquots of these five clones were screened with sera from leprosy patients, close-contacts of leprosy patients and non-contacts (as controls).

14.1 Preliminary Analysis of the Expression Positive gtII/M. leprae Clones

Infection mixtures of E. coli Y1090 cells and phage from each of the five gtII/M. leprae clones were prepared then plated out onto L-Agar plates, which were then incubated at 42°C for 3 hours. The plates were then overlaid with IPTG-soaked nitrocellulose filters and the plates + filters were incubated at 37°C for 3 hours. The filters were then removed from the plates, cut into three pieces (A-, B- and C- fragments) and screened as follows:

(1) the A-fragments were incubated with serum from a lepromatous leprosy patient (LL serum No. 123); biotin conjugated secondary
antibodies and then the streptavidin-biotinylated horseradish peroxidase complex solution;

(2) the E-fragments were incubated with the streptavidin-biotinylated horseradish peroxidase complex solution and

(3) the C-fragments were incubated with serum from a tuberculoid leprosy patient (TT serum No. 29); biotin conjugated secondary antibodies and then the streptavidin-biotinylated horseradish peroxidase complex solution.

Incubation of the filters (i.e. A-, B- and C- fragments) in the peroxidase colour solution revealed strong positive signals on one of the B-fragment filters (i.e. the B-fragment of gtlI/M.leprae clone Y3184). As this fragment had only been screened with the streptavidin-biotinylated horseradish peroxidase complex solution (i.e. no primary antibody treatment), this result implied that gtlI/M.leprae clone Y3184 was expressing a biotinylated M.leprae protein. Strong positive signals were also detected on all of the A-fragment filters and on all of the C-fragment filters (Photograph 18), which suggested that LL serum No. 123 and TT serum No. 29 both contained antibodies to the antigenic M.leprae proteins expressed by clones Y3164, Y3178, Y3179 and Y3180. At this stage, due to the binding of the streptavidin-biotinylated horseradish peroxidase complex solution to the molecule expressed by clone Y3184 (in the absence of primary antibody), it was not possible to decide whether or not these two sera samples also contained antibodies to the biotinylated M.leprae molecule expressed by this clone.
To determine the size of the biotinylated \textit{M. leprae} protein expressed by \textit{gtII/M. leprae} clone Y3184, a crude protein extract of this clone (together with extracts of the four other \textit{gtII/M. leprae} clones) was prepared, as described in section 6.12. An aliquot of each of the five extracts was subjected to SDS-PAGE electrophoresis and the gel was subsequently Western blotted. Incubation of the nitrocellulose filter in the streptavidin-biotinylated horseradish peroxidase complex solution and then in the peroxidase colour solution, confirmed that \textit{gtII/M. leprae} clone Y3184 was expressing a biotinylated \textit{M. leprae} protein (Photograph 19). From its size (larger than 114KD), this expressed molecule is assumed to be a fusion of beta-galactosidase and a biotinylated \textit{M. leprae} molecule. The two low molecular weight bands which also reacted positively with the streptavidin-biotinylated horseradish peroxidase complex solution (tracks 1-6) are biotinylated \textit{E. coli} proteins.
The A-fragments were screened with (i) a 1:1000 dilution of LL serum No. 123; (ii) a 1:2000 dilution of biotin conjugated goat anti-human IgG and (iii) a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution.

The B-fragments were screened with (i) a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution only (i.e. no antibody treatment).

The C-fragments were screened with (i) a 1:1000 dilution of TT serum No. 29; (ii) a 1:2000 dilution of biotin conjugated goat anti-human IgG and (iii) a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution.
The filter was screened with a 1:500 dilution of the streptavidin-biotinylated horseradish peroxidase complex solution and then developed in the peroxidase colour development solution (i.e. no antibody treatment).
14.2 Analysis of the Expression Positive gtII/M. leprae Clones with Alkaline Phosphatase Conjugated Secondary Antibodies

Following the preliminary results in section 14.1 (suggesting that LL serum No.123 and TT serum No.29 contained antibodies to the M. leprae proteins expressed by gtII/M. leprae clones Y3164, Y3178, Y3179 and Y3180) the study was expanded to screen the five gtII/M. leprae clones with many more lepromatous and tuberculoid leprosy sera samples and to also screen these clones with sera from close-contacts of leprosy patients and from non-contacts. As the sera used in section 14.1 had not been absorbed to remove anti-E. coli antibodies, it is possible that the positive signals observed, were simply the results of reactions between E. coli antigens expressed by the five clones and anti-E. coli antibodies present in the two sera samples. Consequently, three control samples (i.e. Controls 1 and 2 were non-recombinant gtII clones, expressing E. coli and phage lambda gtII antigens only and Control 3 was a recombinant gtII/M. bovis BCG clone, expressing an antigenic M. bovis BCG molecule, together with E. coli and phage lambda gtII antigens) were included in the expanded screening study described below.

Four L-Agar plates were each overlaid with a 3ml volume of molten T-top Agar containing a 200μl aliquot of E. coli Y1090 cells. When the overlays had set, single drops (c.10⁴ phage) from the phage stocks of the five gtII/M. leprae clones and the three control samples, were arrayed onto each of the plates (Figure 12). The plates were incubated at 42°C for 3 hours, then overlaid with IPTG-soaked
nitrocellulose filters and incubated at 37°C for 3 hours. Following their removal from the four plates, the filters were each cut into four sections and these were then screened as described in Table 28, i.e. fifteen of the sections were incubated with (i) diluted, human sera samples; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) developed in the phosphatase colour solution. The final filter section was incubated with (i) E.coli absorbed rabbit anti-M. bovis BCG antiserum; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG and then (iii) developed in the phosphatase colour solution. Photographs 20 and 21 show filter sections following their screening with eight different sera samples.

The results (contained in Table 28) revealed that the three Control samples reacted as strongly (positively!) with the fifteen human serum samples, as the five gtII/M.leprae clones, implying that the positive signals were simply the results of anti-E.coli antibodies in the serum samples reacting with the E.coli antigens expressed by each of the clones. It is possible however, that positive M.leprae antigen-antibody reactions were also produced but were masked by the E.coli antigen-antibody reactions but due to the limited volumes of leprosy sera available, I was unable to prepare and use E.coli absorbed sera to rescreen these five gtII/M.leprae clones to test this hypothesis.

Only two positive signals were detected on the filter section screened with E.coli absorbed anti-M.bovis BCG antiserum sample (Filter 4D): (1) the recombinant gtII/M.bovis BCG clone (Control 3), as expected
and (2) gtII/M. leprae clone Y3178. Thus, as stated earlier (section 10.2.1) this rabbit anti-M. bovis BCG antiserum contains antibodies cross-reactive with the 65KD M. leprae antigen expressed by gtII/M. leprae clone Y3178, which is to be expected from the subsequently published sequence similarity between the M. leprae and M. bovis BCG 65KD antigens (Mehra et al., 1986; Thole et al., 1987).
Figure 12: Arraying Clones on L-Agar Plates

Spot 1 = \text{gtII/M. leprae} Clone Y3164
Spot 2 = \text{gtII/M. leprae} Clone Y3178
Spot 3 = \text{gtII/M. leprae} Clone Y3179
Spot 4 = \text{gtII/M. leprae} Clone Y3180
Spot 5 = \text{gtII/M. leprae} Clone Y3184
Spot 6 = Control No. 1 - non recombinant gtII
Spot 7 = Control No. 2 - non recombinant gtII
Spot 8 = Control No. 3 - recombinant \text{gtII/M. bovis} BCG Clone
Table 28: Screening the Expression Positive gtII/M. leprae Clones with Human Sera

<table>
<thead>
<tr>
<th>Filter</th>
<th>Serum</th>
<th>Dilution</th>
<th>gtII/M. leprae clones</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Y3164     Y3178 Y3179 Y3180  Y3184</td>
<td>1  2  3</td>
</tr>
<tr>
<td>2B</td>
<td>LLNo 5</td>
<td>1:1000</td>
<td>+++       +++ +++ +++ +++</td>
<td>++ UR ++</td>
</tr>
<tr>
<td>4A</td>
<td>LLNo 6</td>
<td>1:1000</td>
<td>+++       +++ +++ +++ +++</td>
<td>+++ +++ ++</td>
</tr>
<tr>
<td>2D</td>
<td>LLNo 27</td>
<td>1:1000</td>
<td>+ + + +    + +</td>
<td>+ UR +</td>
</tr>
<tr>
<td>1B</td>
<td>LLNo 34</td>
<td>1:1000</td>
<td>+++       +++ +++ UR +++</td>
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</tr>
<tr>
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<td>++ ++ ++</td>
</tr>
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<td>++ ++ ++</td>
</tr>
<tr>
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<td>++ ++ ++</td>
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<tr>
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<td>+ + +</td>
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<td>++ ++ UR</td>
</tr>
<tr>
<td>1C</td>
<td>CCNo516</td>
<td>1:1000</td>
<td>+++ +++ +++ +++ +++</td>
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<td>NCNoAHM</td>
<td>1:1000</td>
<td>++ ++ ++ ++ UR</td>
<td>++ UR UR</td>
</tr>
<tr>
<td>3B</td>
<td>NCNo EM</td>
<td>1:500</td>
<td>+ + + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>4C</td>
<td>NCNoMTM</td>
<td>1:500</td>
<td>+++ +++ +++ +++ +++</td>
<td>+++ +++ +++</td>
</tr>
<tr>
<td>4D</td>
<td>rabbit anti M. bovis BCG</td>
<td>1:500</td>
<td>- + - - - -</td>
<td>- - ++</td>
</tr>
</tbody>
</table>

The relative strengths of each colour reaction were based on the following scale: +++ = very strong colour reaction, ++ = strong colour reaction, + = less strong colour reaction, - = no colour reaction and UR = unreadable result.

Notes: LL = lepromatous leprosy serum; TT = tuberculoid leprosy serum; CC = close-contact serum; NC = non-contact serum.
Screening the Expression Positive gtII/M.leprae Clones with Human Sera

Filter 3A: screened with serum from a lepromatous leprosy patient (LL No. 123)
Filter 3B: screened with serum from a non-contact (NC No. EM)
Filter 3C: screened with serum from a close-contact of leprosy patients (CC No. 502)
Filter 3D: screened with serum from a lepromatous leprosy patient (LL No. 149)

Filter 4A: screened with serum from a lepromatous leprosy patient (LL No. 6)
Filter 4B: screened with serum from a tuberculoid leprosy patient (TT No. 29)
Filter 4C: screened with serum from a non-contact (NC No. MTM)
Filter 4D: screened with rabbit anti-M.bovis BCG antiserum.
14.3 Western Blot Analysis of Specific Clones with Human Sera Samples

As Western blot analysis is more specific than plaque-screening (individual antigenic bands can be detected), it was hoped that the use of this technique would overcome the need to screen the five expression positive gtlI/M.leprae clones with E.coli absorbed sera samples. Thus, Western blots of these five M.leprae clones (together with E.coli Y1089, as a negative control) were screened with aliquots of serum samples from leprosy patients, in order to determine whether the M.leprae proteins expressed by these clones reacted with anti-M.leprae antibodies or whether the positive-signals detected (following the experiments described in sections 14.1 and 14.2), were simply the results of E.coli antigen-antibody reactions. Similarly, Western blots of the six gtlI/M.bovis BCG clones, expressing antigenic M.bovis BCG proteins (section 11.3) were also screened with serum samples from leprosy patients, to determine if any of these antigenic M.bovis BCG proteins were cross-reactive with M.leprae antibodies.

Crude protein extracts of the five gtlI/M.leprae clones (together with E.coli Y1089 as a negative control) were subjected to SDS-PAGE electrophoresis and then Western blotting. Following blotting, the filters were screened with (i) diluted serum samples (as described in Table 29); (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution. Following development of the filters, each of the five gtlI/M.leprae clones produced an antigenic banding pattern, identical to the pattern produced by the E.coli negative control (data
not shown). The *M. leprae* proteins expressed by these five gtII/*M. leprae* clones were not detected with any of the serum samples used (Table 29). These results confirm that the positive signals observed earlier with these five clones (sections 14.1 and 14.2) were simply due to *E. coli* antigen-antibody reactions.

Crude protein extracts of the six gtII/*M. bovis* BCG clones (532-9, 257-1, 257-2, 306-1, 306-2 and 306-3), together with *E. coli* Y1089 as a negative control were subjected to SDS-PAGE electrophoresis and then Western blotting. Following blotting, the filter was screened with (i) a 1:100 dilution of *E. coli* absorbed, LL serum No. 6; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution. Following development of the filter, five of the gtII/*M. bovis* BCG clones produced antigenic banding patterns identical to the pattern produced by the *E. coli* negative control, however the antigenic *M. bovis* BCG fusion protein expressed by gtII/*M. bovis* BCG clone 532-9 was detected (Photograph 22; marked with — ). Thus gtII/*M. bovis* BCG clone 532-9, is expressing an antigenic *M. bovis* BCG protein, cross-reactive with anti-*M. leprae* antibodies produced by a lepromatous leprosy patient.

To determine whether a relationship could be described between the antibody response to this *M. bovis* BCG antigen and the disease status of individuals, further extracts of this clone were subjected to SDS-PAGE electrophoresis and Western blotting and the resultant filters were then screened with different serum samples. However, as the results in Table 30 show (at this time), no other serum sample has been shown to
contain antibodies to the antigenic \textit{M. bovis} BCG protein expressed by gtII/\textit{M. bovis} BCG clone 532-9.

### Table 29: Results of the Western Blot Analysis of the Expression Positive gtII/\textit{M. leprae} Clones

<table>
<thead>
<tr>
<th>Filter</th>
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<th>Dilution</th>
<th>Clone Y3164</th>
<th>Clone Y3176</th>
<th>Clone Y3179</th>
<th>Clone Y3180</th>
<th>Clone Y3184</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LL No. 6</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>LL No.149</td>
<td>1:1000</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>TT No.29</td>
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<td>No</td>
<td>No</td>
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<td>No</td>
</tr>
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<tr>
<td>5</td>
<td>TT No.85</td>
<td>1:1000</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>CC No.516</td>
<td>1:1000</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
Western Blot Analysis of Specific gtII/\textit{M. bovis} BCG Clones

Track number

1 = \textit{E. coli} Y1089
2 = gtII/\textit{M. bovis} BCG clone 306-1
3 = gtII/\textit{M. bovis} BCG clone 306-2
4 = gtII/\textit{M. bovis} BCG clone 306-3
5 = gtII/\textit{M. bovis} BCG clone 532-9
6 = gtII/\textit{M. bovis} BCG clone 257-1
7 = gtII/\textit{M. bovis} BCG clone 257-2
8 = \textit{E. coli} Y1089

This filter was screened with (i) a 1:100 dilution of \textit{E. coli} absorbed LL serum No. 6; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Table 30: Results Following the Screening of Specific gtl II/M. bovis BCG Clones with Human Sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dilution</th>
<th>Clone 532-9</th>
<th>Clone 257-1</th>
<th>Clone 257-2</th>
<th>Clone 306-1</th>
<th>Clone 306-2</th>
<th>Clone 306-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL No. 6</td>
<td>1:100</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LL No.123</td>
<td>1:100</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>LL No.149</td>
<td>1:1000</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TT No.29</td>
<td>1:100</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TT No.38</td>
<td>1:1000</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TT No.85</td>
<td>1:1000</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CC No.502</td>
<td>1:1000</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CC No.516</td>
<td>1:500</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NC No.MTM</td>
<td>1:500</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Notes: + = expressed antigen detected; - = expressed antigen not detected; ND = not determined.
By screening Western blots, containing a number of mycobacterial species (i.e. *M. bovis* BCG, *M. phlei*, *M. vaccae*, *M. smegmatis* and "*M. lufu""), with sera from tuberculoid and lepromatous leprosy patients; from close-contacts of leprosy patients and from non-contacts, I hoped to identify target antigens for further gene cloning. Moreover, it was also hoped that the antibody responses to these antigens could be used to compare and identify the different classes of leprosy patients. *M. leprae* was not included in the subsequent antisera screening experiments described below because (i) preliminary Western blot analysis with extracts of this species resulted in the detection of antigenic smears, rather than discrete antigenic protein bonds and (ii) because of a shortage of material.

Cultures of five mycobacterial species (listed above) were prepared and harvested (work carried out by J. C. Bunker and Z. Kunze) and the pellets were then resuspended in SDS-PAGE gel loading buffer. Aliquots of each sample were boiled for 5 minutes and then subjected to SDS-PAGE electrophoresis and then Western blotting. The nitrocellulose filters were screened with (i) diluted serum samples (as described in Table 31); (ii) bound primary antibody was detected with phosphatase conjugated secondary antibodies and then (iii) the filters were incubated in the phosphatase colour development solution.

The results showed considerable variation in antigen recognition by the different serum samples moreover, for the majority of the
reactive protein bands there did not appear to be any correlation between the antibody response and the disease status of the individual (Photographs 23-38). A few antigenic bands did however show consistent properties: (1) a 30-40KD "M. lufu" antigen appeared to cross-react with sera from lepromatous leprosy patients only and (2) an c.24KD M. bovis BCG antigen appeared to cross-react with sera from close-contacts and non-contacts (Table 31). Thus, these two antigens may prove useful for diagnosing infected individuals.

It is anticipated that future work will involve (i) screening gtII/"M. lufu" and gtII/M. bovis BCG libraries with appropriate antiserum samples, to isolate clones expressing the 30-40KD "M. lufu" antigen and the c.24KD M. bovis BCG antigen and then (ii) testing the ability of different classes of leprosy patients to recognise the antigens (30-40KD "M. lufu" and c.24KD M. bovis BCG) expressed by the recombinant clones.
Table 31: Detection of Cross-Reactive Antibodies to Two Mycobacterial Antigens

<table>
<thead>
<tr>
<th>Filter</th>
<th>Serum</th>
<th>Dilution</th>
<th>30-40KD &quot;M. lufu&quot; Antigen</th>
<th>24KD M. bovis BCG Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LL No.5</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>LL No.6</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>LL No.27</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>LL No.34</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>LL No.35</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>LL No.123</td>
<td>1:1000</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>LL No.149</td>
<td>1:1000</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>TT No.38</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>TT No.85</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>CC No.502</td>
<td>1:1000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
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<td>1:1000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>CC No.516</td>
<td>1:1000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>NC No.AHM</td>
<td>1:1000</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>NC No.EM</td>
<td>1:1000</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>15</td>
<td>NC No.LMM</td>
<td>1:500</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>16</td>
<td>NC No.MTM</td>
<td>1:500</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Photograph 23: Western Blot Analysis with LL serum No.5

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of LL serum No. 5; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.

Photograph 24: Western Blot Analysis with LL serum No.6

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of LL serum No. 6; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Photograph 25: Western Blot Analysis with LL serum No. 27

Track number
1 = M.bovis BCG
2 = M.phlei
3 = M.vaccae
4 = M.smegmatis
5 = "M.lufu"

This filter was screened with (i) a 1:1000 dilution of LL serum No. 27; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.

Photograph 26: Western Blot Analysis with LL serum No. 34

Track number
1 = M.bovis BCG
2 = M.phlei
3 = M.vaccae
4 = M.smegmatis
5 = "M.lufu"

This filter was screened with (i) a 1:1000 dilution of LL serum No. 34; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Photograph 27: Western Blot Analysis with LL serum No. 35

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of LL serum
No. 35; (ii) a 1:1000 dilution of
alkaline phosphatase conjugated
goat anti-human IgG and then
(iii) incubated in the phosphatase
colour development solution.

Photograph 28: Western Blot Analysis with LL serum No. 123

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of LL serum
No. 123; (ii) a 1:1000 dilution of
alkaline phosphatase conjugated
goat anti-human IgG and then
(iii) incubated in the phosphatase
colour development solution.
Western Blot Analysis with LL serum No. 149

Track number:
1 = *M. bovis* BCG
2 = *M. phlei*
3 = *M. vaccae*
4 = *M. smegmatis*
5 = "M. lufu"

This filter was screened with:
(i) a 1:1000 dilution of LL serum No. 149;
(ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then
(iii) incubated in the phosphatase colour development solution.
Photograph 30: Western Blot Analysis with TT serum No. 38

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of TT serum No. 38; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.

Photograph 31: Western Blot Analysis with TT serum No. 85

Track number
1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of TT serum No. 85; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Photograph 32: Western Blot Analysis with CC serum No. 502

Track number:

1 = *M. bovis* BCG
2 = *M. phlei*
3 = *M. vaccae*
4 = *M. smegmatis*
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of CC serum No. 502; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.

Photograph 33: Western Blot Analysis with CC serum No. 505

Track number:

1 = *M. bovis* BCG
2 = *M. phlei*
3 = *M. vaccae*
4 = *M. smegmatis*
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of CC serum No. 505; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Photograph 34: Western Blot Analysis with CC serum No. 516

Track number:
1 = *M. bovis* BCG
2 = *M. phlei*
3 = *M. vaccae*
4 = *M. smegmatis*
5 = "M. lufu"

This filter was screened with (i) a 1:1000 dilution of CC serum No. 516; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then (iii) incubated in the phosphatase colour development solution.
Photograph 25: Western Blot Analysis with NC serum No. AHX

Photograph 26: Western Blot Analysis with NC serum No. EM

This filter was screened with
(i) a 1:1000 dilution of NC serum
No. AHX; (ii) a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgG and then
(iii) incubated in the phosphatase colour development solution.
Photograph 37:

Western Blot Analysis with NC serum No. LMM

Track number:

1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of NC serum
No. LMM; (ii) a 1:1000 dilution of alkaline phosphatase conjugated
goat anti-human IgG and then
(iii) incubated in the phosphatase
colour development solution.

Photograph 38:

Western Blot Analysis with NC serum No. MTM

Track number:

1 = M. bovis BCG
2 = M. phlei
3 = M. vaccae
4 = M. smegmatis
5 = "M. lufu"

This filter was screened with
(i) a 1:1000 dilution of NC serum
No. MTM; (ii) a 1:1000 dilution of alkaline phosphatase conjugated
goat anti-human IgG and then
(iii) incubated in the phosphatase
colour development solution.
To date DNA sequence data of mycobacterial genes is restricted to three genes, the genes expressing: (i) the 65KD *M. leprae* antigen (Mehra et al., 1986); (ii) the 65KD *M. tuberculosis* antigen (Shinnick, 1987) and (iii) the 65KD *M. bovis* BCG antigen (Thole et al., 1987). Thus, following the results from section 14, showing that serum from a lepromatous leprosy patient (LL serum No. 6) contained antibodies to the *M. bovis* BCG antigen expressed by gtII/ *M. bovis* BCG clone 532-9, I considered this *M. bovis* BCG gene a suitable candidate for sequencing.

Of the methods developed for determining the nucleotide sequence of a cloned DNA fragment (Gilbert, 1981; Sanger, 1981), the chain termination sequencing method of Sanger et al. (1977) offers advantages in terms of the rapidity and simplicity of its protocol. This technique is based upon the use of dideoxynucleotide analogues which are randomly incorporated into a growing DNA strand to give specific chain termination.

*E. coli* DNA polymerase I (Klenow fragment), if given a single-stranded DNA template; a primer with a 3'-hydroxyl group and all four deoxynucleoside triphosphates (dNTPs), has the ability to synthesise a DNA strand complementary to that of the single-stranded template. In chain termination sequencing reactions, the primer is annealed to its complementary sequence on the single-stranded DNA template, then the Klenow fragment extends the primer in the 5' to 3' direction, by adding
a single nucleotide at a time until a complete complementary strand has been synthesised (producing a complete double stranded DNA molecule). The Klenow fragment also has the ability to incorporate a dideoxynucleotide (lacking a 3'-hydroxyl group), instead of a deoxynucleotide and whenever this happens in the growing DNA strand, chain termination will result (as there is no 3'-hydroxyl group for formation of the next phosphodiester bond).

During sequence analysis four separate reactions are carried out. Each reaction is supplied with all four dNTPs (i.e. dATP, dCTP, dGTP and dTTP) but only one of the four ddNTPs (i.e. ddATP or ddCTP or ddGTP or ddTTP). Thus in the case of the reaction with ddATP, when the Klenow fragment comes to incorporate deoxyadenosine monophosphate, it has the choice between dATP and ddATP as the substrate. If the dideoxynucleotide is incorporated then the reaction stops by chain termination. However, if the deoxynucleotide is incorporated, the reaction continues and the enzyme is again presented with a choice between dATP and ddATP when it comes to incorporate the next adenosine moiety. By carefully controlling the ratio between dATP and ddATP, incorporation of the dideoxynucleotide and hence chain termination, will be random. The end result of such a reaction is a set of DNA fragments of different lengths, each terminated at the 3'-end with a dideoxyadenosine monophosphate.

By carrying out four separate reactions, each with only one of the four ddNTPs, four separate sets of fragments are formed, each set terminated with either ddT, ddC, ddG or ddA. The 5'-ends share a common
starting point, the primer. The development of high definition, thin
polyacrylamide gels (Sanger and Coulson, 1978) has made possible the
separation of DNA fragments differing in size by only a single
nucleotide; thus, these dideoxy terminated fragments can be accurately
size-separated on such a gel. If one of the dNTPs in the reaction
mixture is radioactively labelled at the alpha-position, the size
separated fragments can be detected by autoradiography. Each band on
the gel arises from termination at a specific point along the chain and
from this the base sequence can be deduced.

The sequence is read from the autoradiograph by recording the
positions of bands across the four tracks, each representing the
positions of ddA, ddC, ddG or ddT terminated fragments. The smallest
fragment (that is the first band at the bottom of the gel) is located
and the track (A, C, G or T) in which this band appears is recorded.
Moving up the gel, from track to track, the sequence of the DNA fragment
is recorded in a 5' to 3' direction from the primer.

16.1 Cloning gtlII/M.bovis BCG clone 532-9 Insert DNA into the Vector
M13mp-2

Recombinant phage DNA was extracted from an amplified phage
stock of gtlII/M.bovis BCG clone 532-9, in accordance with the procedure
described in section 6.4.1 and an aliquot (10 \( \mu \)g) was then digested with
EcoRI at 37°C overnight. The digest was then subjected to agarose gel
electrophoresis and the 3.5Kb M.bovis BCG DNA insert fragment was
isolated (section 6.5.6).
A 1µg aliquot of the cloning vector M13mp-9 was digested with EcoRI and then phosphatase treated (with CIAP) and then vector (30ng) and insert DNA (150ng) were ligated together (section 6.17.1). Five control ligations were also prepared (see Table 32), to allow the efficiency of the subsequent transformation reaction to be assessed (below).

Following overnight incubation at 12°C, the M13mp-9/insert DNA ligation reaction and the five control ligations were used to transform competent E.coli JM101 cells. The transformation mixtures were prepared, as described in section 6.17.2, then plated out onto L-Agar plates containing IPTG and X-gal. The plates were then incubated at 37°C overnight.

When plated out onto media containing IPTG and X-gal, non-recombinant M13 phage appear as blue plaques and recombinant phage appear as colourless or "white" plaques. This discrimination is based upon the presence or absence of the enzyme beta-galactosidase. E.coli cells expressing inactive beta-galactosidase (i.e. E.coli JM101 cells) but infected with any of the M13 cloning vectors (i.e. intact vector molecules) will, in the presence of the lac operon inducer IPTG, produce functional beta-galactosidase. These cells will subsequently hydrolyse the substrate X-gal, producing a blue dye. Insertion of foreign DNA into a suitable cloning site within the M13 vector interferes with the production of functional beta-galactosidase in infected E.coli JM101 cells and as a consequence recombinant phage appear as colourless.
plaques on the uninfected *E. coli* lawn (in contrast to the blue plaques produced by non-recombinant phage).

Table 32 lists the results of the six transformation reactions. The results of the five control experiments indicate that (i) the production of the M13mp-9 vector had been very good; (ii) the T4-DNA ligase was working and (iii) competent *E. coli* JM101 cells had been produced and the transformation reaction had worked. Although the number of plaques in the test transformation is small, the absence of "white" plaques in the controls makes it highly likely that the nine "white" plaques in the test reaction are genuine recombinants.

<table>
<thead>
<tr>
<th>Cloning Experiment</th>
<th>Plaque Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp-9 (30ng), EcoRI-digested, CIAP-treated + gIII/<em>M. bovis</em> BCG clone 532-9 + insert DNA (150ng) + ligase</td>
<td>9 6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. M13mp-9(30ng), EcoRI-digested, CIAP-treated + ligase</td>
<td>4</td>
</tr>
<tr>
<td>2. M13mp-9(30ng), EcoRI-digested, CIAP-treated</td>
<td>0</td>
</tr>
<tr>
<td>3. M13mp-9(30ng), EcoRI-digested + ligase</td>
<td>133</td>
</tr>
<tr>
<td>4. M13mp-9(30ng), EcoRI-digested</td>
<td>9</td>
</tr>
<tr>
<td>5. M13mp-9 (1ng), uncut</td>
<td>103</td>
</tr>
</tbody>
</table>
16.2 DNA Sequence Data

Each of the nine "white" plaques (i.e. M13mp-9 clones 1-9) were picked from the L-Agar plate (section 16.1) and used to inoculate 1.5ml volumes of diluted E.coli JM101 cells, which were then incubated at 37°C for 6-8 hours. Following incubation at 37°C, the nine samples were pelleted and double-stranded DNA (dsDNA) was extracted from the cell pellets in accordance with the method described in section 6.3.2 and single-stranded DNA (ssDNA) was extracted from the nine supernatants (section 6.17.3).

Initially, aliquots of the nine dsDNA samples were digested with EcoRI to verify that each of the clones contained the 3.5Kb M. bovis BCG DNA insert fragment. The digests were analysed on 1% agarose gels and three DNA bands were detected in each track: band 1, the 3.5Kb insert DNA fragment; band 2, linearised M13mp-9 vector DNA and band 3, undigested dsDNA (i.e. vector + insert DNA). Photograph 39 shows the restriction analysis of five clones.

There are two possible orientations for the insertion of the 3.5Kb insert DNA fragment in the M13mp-9 cloning vector and these were distinguished by the location of asymmetrically placed SaI restriction sites within the insert fragment, relative to the SaI site within the M13mp-9 vector. Aliquots of the nine dsDNA samples were digested with SaI, then analysed on a 1% agarose gel. Photograph 40 shows the SaI digests of four dsDNA samples (M13mp-9 clones 1-4), revealing two
digestion patterns, thus M13mp-9 clones 1, 2 and 4 had the 3.5Kb insert DNA fragment cloned in one orientation and M13mp-9 clone 3 had the insert cloned in the reverse orientation.

The orientation of the 3.5Kb insert DNA fragment in gtII/M. bovis BCG clone 532-9 had not been determined (consequently the orientation of the coding region was unknown) and it was therefore necessary to sequence the 3.5Kb insert in both orientations. M13mp-9 clones 1 and 3 were selected and ssDNA aliquots of both were subjected to the Sanger "dideoxy" sequencing method (Sanger et al., 1977), as described in section 6.17.4. Figures 13 and 14 list the sequence data collected from the two sequencing reactions, i.e. 227 nucleotides were determined from M13mp9 clone 1 and 241 nucleotides were determined from M13mp-9 clone 3.

Figure 13: Partial Nucleotide Sequence of M13mp-9 Clone 1

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>GTAATCGGCC</td>
</tr>
<tr>
<td>20</td>
<td>CAAGGAGTC</td>
</tr>
<tr>
<td>30</td>
<td>AATCAACGNC</td>
</tr>
<tr>
<td>40</td>
<td>ATCACCGAGG</td>
</tr>
<tr>
<td>50</td>
<td>AGACCCGGCGC</td>
</tr>
<tr>
<td>60</td>
<td>CCAGATCTCA</td>
</tr>
<tr>
<td>70</td>
<td>TCGAGGACGA</td>
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<td>CGGCAGCGTG</td>
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<td>90</td>
<td>TTCGTCGGGG</td>
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<tr>
<td>100</td>
<td>CACGANNCGT</td>
</tr>
<tr>
<td>110</td>
<td>GGNGNAGCCG</td>
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<td>120</td>
<td>ATCGACGGAT</td>
</tr>
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<td>TNTGATGTA</td>
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<tr>
<td>170</td>
<td>AGAAAGGATT</td>
</tr>
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<td>TNGGNGGTG</td>
</tr>
<tr>
<td>190</td>
<td>TGTGINTPNG</td>
</tr>
<tr>
<td>200</td>
<td>GGTCAGGGGT</td>
</tr>
<tr>
<td>210</td>
<td>GTGAATTCGA</td>
</tr>
<tr>
<td>220</td>
<td>ANNTGGAGGA</td>
</tr>
</tbody>
</table>

(K = ambiguous nucleotides)
Comparison of the nucleotide sequences in Figures 13 and 14 with the three published sequences, i.e. the sequences for the genes expressing: (1) the 65KD \textit{M. leprae} antigen (Mehra et al., 1986); (ii) the 65KD \textit{M. tuberculosis} antigen (Shinnick, 1987) and (iii) the 65KD \textit{M. bovis} BCG antigen (Thole et al., 1987) revealed no significant homology between my sequences and the published sequences, thus \textit{gtII/M. bovis} clone 532-9 is not expressing the 65KD \textit{M. bovis} BCG antigen.

It is anticipated that future work will involve: (1) sequencing the remainder of the 3.5Kb insert fragment of \textit{gtII/M. bovis} BCG clone 532-9 (to date only c. 10% of this insert fragment has been sequenced); (ii) the preparation of a DNA probe (using the 3.5Kb insert fragment of clone 532-9) to screen the \textit{gtII/M. bovis} BCG library to isolate a clone containing the complete gene; (iii) cloning the complete gene into an expression vector to determine the size of the expressed antigen and
(iv) characterisation of the expressed antigen with the monoclonal antibodies which are currently available, to determine if this antigen is the *M. bovis* BCG equivalent of any of the cloned *M. leprae* and *M. tuberculosis* antigens.
Photograph 32:  EcoRI Digestion of Five dsDNA Samples

Track number

1 = EcoRI digest of M13mp-9 clone 1 dsDNA
2 = EcoRI digest of M13mp-9 clone 2 dsDNA
3 = EcoRI digest of M13mp-9 clone 3 dsDNA
4 = EcoRI digest of M13mp-9 clone 4 dsDNA
5 = EcoRI digest of M13mp-9 clone 5 dsDNA
7 = HindIII digest of Lambda DNA

Notes: band 1 = the 3.5Kb insert DNA fragment; band 2 = linearised M13mp-9 vector DNA; band 3 = undigested dsDNA.
Photograph 40: Sall Digestion of Four dsDNA Samples

Track number:

1 = HaeIII digest of φX174 DNA
2 = HindIII digest of Lambda DNA
3 = Sall digest of M13mp-9 clone 1 dsDNA
4 = Sall digest of M13mp-9 clone 2 dsDNA
5 = Sall digest of M13mp-9 clone 3 dsDNA
6 = Sall digest of M13mp-9 clone 4 dsDNA
There were two objectives to this study, the first was to confirm the suitability of \textit{E. coli} as a host for the cloning and expression of mycobacterial genes [doubts had been raised concerning the use of \textit{E. coli} when Clark-Curtiss et al. (1985) reported their inability to obtain recombinant mycobacterial clones able to complement mutations in \textit{E. coli} cells]. The second objective was to determine whether the \textit{M. leprae}-infection (disease) status of individuals could be elicited, through the detection of antibodies to specific mycobacterial antigens, in their antiserum.

17.1 The Recognition of \textit{M. bovis} BCG Promoters by \textit{E. coli} RNA Polymerase

Gene expression is dependent upon (i) recognition of the gene promoter site by the enzyme RNA polymerase; (ii) synthesis of RNA transcripts, complementary to the DNA message encoded by the gene in question and (iii) translation of the RNA transcripts (by ribosomes) into the product encoded by the original DNA message. Consequently, three main possibilities exist which could account for the failure of Clark-Curtiss et al. (1985), to obtain mycobacterial gene expression in \textit{E. coli}: (1) the \textit{E. coli} RNA polymerase enzyme was unable to recognise mycobacterial gene promoter sites and thus mycobacterial genes were not transcribed into RNA transcripts; (2) mycobacterial genes may have been transcribed but the \textit{E. coli} ribosomes were unable to recognise and bind to the mycobacterial ribosome-\textit{binding sites}, preventing the translation
of the RNA transcripts into the mycobacterial gene products or (3) transcription and translation may have occurred but either the level of expression of mycobacterial gene products was too low or the expressed molecules were non-functional (the detection of expressed molecules able to complement mutations in the host cell is dependent upon these expressed molecules being functional). Accordingly, my first experiments were designed and undertaken to show that mycobacterial promoters could be recognised and utilised by E. coli RNA polymerase.

The results of the promoter-probe experiments (section 8.1 and 8.2), whilst showing that twice as many homologous (i.e. E. coli HB101) as heterologous promoters (i.e. M. bovis BCG) were recognised by E. coli RNA polymerase, also revealed that a significant proportion of M. bovis BCG promoters were functional in E. coli. These results confirmed that (in this respect at least) E. coli is a suitable host for obtaining the expression of mycobacterial genes and moreover implied that those promoters recognised by E. coli RNA polymerase shared a high degree of homology with E. coli promoter sequences. Kieser et al. (1986) however, have shown that mycobacterial DNA can be cloned more efficiently in S. lividans than in E. coli (these authors reported that S. lividans uses M. bovis BCG transcriptional and translational signals as efficiently as its own signals), thus S. lividans appears to be a better host than E. coli for the cloning and expression of mycobacterial genes.

Three of the most well-characterised mycobacterial genes, expressing (1) a 65KD M. leprae antigen; (2) a 65KD M. tuberculosis antigen and (3) a 65KD M. bovis BCG antigen (each of whose transcription
and translation signals are functional in *E. coli*, have recently been sequenced by Mehra et al. (1986), Shinnick (1987) and Thole et al. (1987), respectively. Moreover, I have identified (within each of these published sequences) nucleotide sequences, highly homologous to (i) the -10 and -35 consensus sequences of typical *E. coli* K-12 promoters (Hawley and McClure, 1983) and (ii) *E. coli* K-12 Shine-Dalgarno sequences (Shine and Dalgarno, 1974). Table 33 compares the putative mycobacterial transcription and translational signals of these three mycobacterial genes with typical *E. coli* signals and furthermore, states the position of these putative mycobacterial signals in relation to the ATG translation codon of the respective genes. The extent of similarity between the putative mycobacterial control signals and the typical *E. coli* control signals is high: (i) the "Shine-Dalgarno" sequences show a 6 out of 7 basepair (bp) identity with the *E. coli* consensus sequence; (ii) the "-10 region" sequences of the *M. tuberculosis* and *M. bovis* BCG genes show a 4 out of 6 bp identity with the *E. coli* consensus sequence; (iii) the *M. leprae"-10 region" sequence shows a 3 out of 6bp identity with the *E. coli* consensus sequence and (iv) the "-35 region" sequences show a 4 out of 6bp identity with the *E. coli* consensus sequence, thus it is perhaps not surprising that these three mycobacterial genes are expressed in *E. coli*.

Since the greater proportion of mycobacterial genes appear not to be expressed in *E. coli* (section 8.2), the mycobacterial promoter sequences described in Table 33 may not be representative of mycobacterial promoters. At this time there is no DNA sequence data available for mycobacterial promoters considered to be non-functional,
thus it is possible to speculate that Mycobacteria, like Streptomyces
(Westpheling et al., 1985) and Bacillus (Doi and Wang, 1986), may
contain different classes of promoters and furthermore in common with
Streptomyces (Jaurin and Cohen, 1984) and Bacillus (Moran et al., 1982)
one of the mycobacterial RNA polymerases recognises R.coli-type
promoters.
Table 33: Putative Transcription and Translation Signals of Three Mycobacterial Genes

<table>
<thead>
<tr>
<th>Putative Mycobacterial Control Sequences</th>
<th>M. leprae gene (expressing a 65KD antigen)</th>
<th>M. tuberculosis gene (expressing a 65KD antigen)</th>
<th>M. bovis BCG gene (expressing a 65KD antigen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical E. coli K-12 Control Sequences</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shine-Dalgarno Consensus Sequence: AGGAGGA</td>
<td>CCGAGGA (11bp)</td>
<td>CCGAGGA (11bp)</td>
<td>CCGAGGA (11bp)</td>
</tr>
<tr>
<td>-10 region Consensus Sequence: TATAAT</td>
<td>CTTCA T (26bp)</td>
<td>TTTCA T (26bp)</td>
<td>TTTCA T (26bp)</td>
</tr>
<tr>
<td>-35 region Consensus Sequence: TTGACA</td>
<td>TTGTCA G (49bp)</td>
<td>TTGCG C G (49bp)</td>
<td>TTGCG C G (50bp)</td>
</tr>
</tbody>
</table>

Notes: The figures in brackets denote the position (in base pairs) of the putative mycobacterial signals, upstream of their respective translation start codons (ATG).
A variety of vector systems have been employed with the aim of introducing mycobacterial genes into \textit{E. coli} and obtaining their expression. Clark-Curtiss et al. (1985) initially cloned \textit{M. vaccae} and \textit{M. leprae} DNA restriction fragments into the vectors pBR322 and pHC79 but were not able to detect recombinant clones expressing mycobacterial gene products. These workers then ligated \textit{M. leprae} DNA restriction fragments into the plasmid expression vector pYA626, which contained the \textit{Streptococcus mutans} aspartate beta-semialdehyde dehydrogenase promoter (Jacobs et al., 1986), thus \textit{M. leprae} genes were placed under the control of a promoter, efficiently recognised in \textit{E. coli}. Their subsequent screening of their pYA626/\textit{M. leprae} clones resulted in the detection of a clone expressing a 46KD \textit{M. leprae} polypeptide.

Thole et al. (1985) used the phage Lambda vector EMBL3 to prepare an \textit{M. bovis} BCG library and their subsequent screening of these clones, by Western blotting, resulted in the detection of clones expressing \textit{M. bovis} BCG antigens (i.e. antigens 64KD, 70KD, 95KD, >100KD, 90KD and 30KD in size). These authors however, further reported that the recombinant clones expressing the >100KD, 90KD and 30KD antigens did not give reproducible results and it was proposed that this was a consequence of the unstable nature of these proteins in the \textit{E. coli} host (unusual polypeptides are efficiently recognised and degraded in \textit{E. coli}).
A number of workers have reported their use of the phage lambda vector gtII, for the preparation of mycobacterial gene libraries. This vector was specifically constructed to permit the insertion of foreign DNA into the beta-galactosidase structural gene (lacZ), under the control of the lac promoter thus: (1) mycobacterial genes can be placed under the control of an E. coli promoter, overcoming the need for gene expression to be dependent upon the recognition of mycobacterial promoters by E. coli RNA polymerase and (2) mycobacterial genes can be expressed as fusions with beta-galactosidase, which enhances the stability of the expressed antigens and reduces the likelihood that these mycobacterial proteins will be degraded by E. coli proteases. Young and his co-workers (1985a; b) prepared gtII/M. tuberculosis and gtII/M. leprae libraries and subsequently identified recombinant clones expressing the following antigenic molecules i.e. 12KD, 18KD, 28KD, 36KD and 65KD M. leprae antigens and 14KD, 19KD and 65KD M. tuberculosis antigens. Other workers have similarly employed gtII for the expression of mycobacterial antigens: (1) Husson and Young (1987), Young et al. (1987a) and Shinnick et al. (1987) have reported the detection of recombinant clones expressing 12KD and 71KD M. tuberculosis antigens and (2) Collins et al. (1987) have reported the detection of recombinant gtII clones expressing biotinylated mycobacterial proteins.

In this thesis, mycobacterial gene libraries were prepared with the following vectors: pBR322, pUC13, EMBL4 and gtII. The plasmid vector pBR322 and the phage lambda vector EMBL4 were used, in the hope of detecting mycobacterial genes undergoing expression from their own promoters (i.e. neither of these two vectors is an expression vector).
and the plasmid vector pUC13 and the phage lambda vector gtII were used to place the mycobacterial genes under the control of a strong E. coli promoter, thus eliminating the dependence of mycobacterial gene expression on E. coli RNA polymerase recognition of mycobacterial promoters. Table 34 lists the recombinant clones identified during this thesis, together with details of their respective gene products.

As Table 34 shows, I was unable to detect pBR322 and pUC13 clones expressing M. leprae antigens. However, I believe that this failure was simply a consequence of the screening procedure employed, i.e. too few clones were tested (only 768 pBR322/M. leprae and only 1632 pUC13/M. leprae clones) and moreover these clones were only screened with three anti-M. leprae monoclonal antibodies. I was also unable to detect EMBL4 clones expressing M. bovis BCG antigens and believe that the in situ screening procedure used to screen the EMBL4/M. bovis BCG library was not sensitive enough to detect expressed antigens. Despite the availability of increased numbers of anti-M. leprae monoclonal antibodies and Thole et al's (1985) successful use of Western blotting to detect EMBL3/M. bovis BCG clones expressing M. bovis BCG antigens (these authors had previously failed to detect recombinant EMBL3 clones expressing M. bovis BCG antigens by the in situ screening procedure), I did not re-screen the pBR322/M. leprae, pUC13/M. leprae or the EMBL4/M. bovis BCG libraries as I considered I had obtained sufficient recombinant clones expressing mycobacterial antigens, from my other libraries (Table 34).
### Table 34: Summary of Recombinant Clones Identified During This Thesis

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Mycobacterial DWA</th>
<th>Vector</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M. bovis BCG</td>
<td>pBR322</td>
<td>65KD antigenic protein</td>
</tr>
<tr>
<td>E</td>
<td>M. bovis BCG</td>
<td>pBR322</td>
<td>65KD antigenic protein</td>
</tr>
<tr>
<td>531-6</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>c.85KD biotinylated protein</td>
</tr>
<tr>
<td>532-9</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>antigenic fusion protein</td>
</tr>
<tr>
<td>257-1</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>antigenic fusion protein</td>
</tr>
<tr>
<td>257-2</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>c.80KD antigenic protein</td>
</tr>
<tr>
<td>306-1</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>antigenic fusion protein</td>
</tr>
<tr>
<td>306-2</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>antigenic fusion protein</td>
</tr>
<tr>
<td>306-3</td>
<td>M. bovis BCG</td>
<td>gtII</td>
<td>antigenic fusion protein</td>
</tr>
<tr>
<td>SAB-2</td>
<td>M. leprae</td>
<td>gtII</td>
<td>c.85KD biotinylated protein</td>
</tr>
<tr>
<td>SAB-7</td>
<td>M. leprae</td>
<td>gtII</td>
<td>c.85KD biotinylated protein</td>
</tr>
<tr>
<td>SAB-3</td>
<td>M. leprae</td>
<td>gtII</td>
<td>c.85KD biotinylated protein</td>
</tr>
<tr>
<td>ML-301</td>
<td>M. leprae</td>
<td>gtII</td>
<td>65KD antigenic protein</td>
</tr>
<tr>
<td>ML-302</td>
<td>M. leprae</td>
<td>gtII</td>
<td>65KD antigenic protein</td>
</tr>
</tbody>
</table>
17.3 Cross-reactivity of the 65KD Mycobacterial Protein

To date, despite results suggesting that a significant proportion of mycobacterial promoters are functional in E. coli (section 8.2), the literature confirms that only a small number of mycobacterial genes have been identified, whose promoters (and translation signals) are able to function in E. coli (Table 35). A slightly larger group of mycobacterial genes have also been identified, with functional translation signals (although experimentation has still to determine whether the promoter signals of these genes are also functional in E. coli). Two reasons however, can account for this lack of corroboration with my earlier results: (1) the majority of workers have used the phage lambda vector gtII to prepare mycobacterial gene libraries and as the foreign DNA is placed under the control of the E. coli lacZ promoter, this cloning vector is biased towards the expression of foreign proteins as fusion proteins with betagalactosidase and (2) monoclonal antibodies, against only twelve mycobacterial antigens, have been used to screen the majority of the gtII libraries described in the literature, thus only a limited number of mycobacterial promoters have been tested for their ability to function in E. coli.

As Table 35 shows, a number of workers (including myself) identified recombinant clones expressing 64-65KD proteins from four different mycobacterial species. I subsequently obtained samples of the clones expressing the following three proteins: the 65KD M. leprae protein (Young et al., 1985b); the 64KD M. bovis BCG protein (Thole et
al., 1985) and the 64KD M. fortuitum protein (Labidi et al., 1985), to investigate the possible existence of a relationship between each of these three mycobacterial proteins and my own 65KD M. bovis BCG protein (expressed by pBR322/M. bovis BCG clone A). With the aid of an absorbed antiserum solution, I was able to demonstrate that Thole et al's 64KD M. bovis BCG protein and my own 65KD M. bovis BCG protein were the same molecule (section 10.2.1). Furthermore I also demonstrated that antibodies recognising the 65KD M. bovis BCG protein were also able to recognise Young et al's 65KD M. leprae protein, which implied that these two proteins were cross-reactive (i.e. shared common epitopes). However, I was unable to show any cross-reactivity between the 64KD M. fortuitum protein of Labidi et al, and either the 65KD M. bovis BCG protein or the 65KD M. leprae protein (sections 10.2.1 and 10.2.2) and conclude that this M. fortuitum protein is not related to the similarly sized M. bovis BCG and M. leprae proteins, employed in this screening experiment.

Other workers have also investigated the immunological relatedness between the different 65KD mycobacterial proteins. Husson and Young (1987), using eleven anti-65KD monoclonal antibodies, reported that the 65KD M. leprae and M. tuberculosis proteins shared seven epitopes. This high degree of cross-reactivity between these two proteins was further confirmed recently, following publication of the DNA and amino acid sequences of the 65KD M. leprae antigen (Mehra et al., 1986) and the 65KD M. tuberculosis antigen (Shinnick, 1987). Comparison of the two amino acid sequences revealed that these two proteins share c.95% homology (these two proteins only differ in 26
amino acid substitutions). Comparison of the DNA and amino acid sequences of the 65KD *M. tuberculosis* protein (Shinnick, 1987) and the 64KD *M. bovis* BCG protein (Thole et al., 1987) has also become possible recently, revealing that these two proteins are 100% homologous. Moreover these results reveal that this 65KD protein is a highly conserved protein. Furthermore, with (1) Thole et al. (1985) demonstrating the presence of protein, in each of nine mycobacterial species that they tested, able to cross-react with antibodies against their 64KD *M. bovis* BCG protein and (2) Buchanan et al. (1987) screening 24 mycobacterial species with fourteen monoclonal antibodies, each recognising a different epitope within the 65KD *M. leprae* protein and finding that thirteen of these monoclonal antibodies recognised epitopes on as few as eight or as many as 24 of the test mycobacterial species, the 65KD mycobacterial protein is considered to be a common mycobacterial protein.
<table>
<thead>
<tr>
<th>Mycobacterial Species</th>
<th>Size of Expressed Protein</th>
<th>Transcription Signals</th>
<th>Translation Signals</th>
<th>Reference Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. bovis BCG</td>
<td>64KD</td>
<td>YES</td>
<td>YES</td>
<td>Thole et al., 1985</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>64KD</td>
<td>YES</td>
<td>YES</td>
<td>Labidi et al., 1985</td>
</tr>
<tr>
<td>M. leprae</td>
<td>65KD</td>
<td>YES</td>
<td>YES</td>
<td>Mehra et al., 1986</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>65KD</td>
<td>YES</td>
<td>YES</td>
<td>Husson &amp; Young, 1987</td>
</tr>
<tr>
<td>M. leprae</td>
<td>46KD</td>
<td>No</td>
<td>YES</td>
<td>Jacobs et al., 1986</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>14KD</td>
<td>?</td>
<td>YES</td>
<td>Young et al., 1987a</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>19KD</td>
<td>YES</td>
<td>YES</td>
<td>Shinnick et al., 1987</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>71KD</td>
<td>?</td>
<td>YES</td>
<td>Young et al., 1987a</td>
</tr>
<tr>
<td>M. bovis</td>
<td>61KD</td>
<td>YES</td>
<td>YES</td>
<td>Collins et al., 1987</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>65KD</td>
<td>?</td>
<td>YES</td>
<td>This thesis</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>80KD</td>
<td>?</td>
<td>YES</td>
<td>This thesis</td>
</tr>
<tr>
<td>M. bovis BCG</td>
<td>85KD</td>
<td>No</td>
<td>YES</td>
<td>This thesis</td>
</tr>
<tr>
<td>M. leprae</td>
<td>85KD</td>
<td>-</td>
<td>YES</td>
<td>This thesis</td>
</tr>
</tbody>
</table>

? these transcription signals are possibly functional in E. coli.
- experimentation has yet to determine whether these transcription signals are functional in E. coli.
17.4 Biological Functions of Some Cloned Mycobacterial Antigens

Recently a possible biological function has been deduced for the 65KD common mycobacterial antigen by Young et al. (1987b). These authors identified a 125 amino acid sequence within the 65KD mycobacterial protein which showed 65% homology with the amino acid sequence derived from the E.coli ams gene. Chanda et al. (1985) had previously shown that a mutation in the ams gene resulted in an extended half-life for messenger RNA, thus Young and his colleagues have suggested that the 65KD mycobacterial protein may be involved in the processing of a messenger RNA molecule.

In sections 10.3 and 13.3, I described recombinant clones expressing biotinylated M.hovis BCG and M.leprae proteins and moreover (section 11.2) described the presence of biotinylated proteins in each of five mycobacterial species, i.e. each of these five species contained a major biotinylated protein of c.65KD, together with two minor biotinylated proteins of c.85KD and c.120KD. Biotinylated enzymes are invariably carboxylases, with the biotin molecule serving as a covalently bound "CO₂ carrier" for reactions in which: (i) CO₂ is fixed into an acceptor molecule (carboxylase reaction); (ii) a carboxyl group is transferred from a donor to an acceptor molecule (transcarboxylase reaction) or (iii) a carboxyl group is removed from a donor molecule as CO₂ (decarboxylase reaction) (Wood and Barden, 1977). From the work of Erfle (1973) and Henrikson and Allen (1979), I believe biological functions can be assigned to a number of the biotinylated mycobacterial proteins I have detected during this project. These authors (Erfle;
Henrikson and Allen) reported the purification of acetyl-CoA carboxylase from *M. smegmatis*, a biotinylated enzyme involved in the first step of fatty acid biosynthesis (catalysing the synthesis of malonyl-CoA from acetyl-CoA). Furthermore, this enzyme was shown to contain two types of subunit proteins, with molecular weights of 64KD and 57KD, arranged in an A4B4 conformation, with biotin shown to be associated with the heavier subunit only. From these results, I believe that (i) the major biotinylated protein (c.65KD), present in each of the five mycobacterial species tested (section 11.2), may correspond to the 64KD subunit protein of mycobacterial acetyl-CoA carboxylase; (ii) the c.85KD biotinylated proteins may represent a second mycobacterial carboxylase enzyme subunit and (iii) the c.120KD biotinylated proteins may simply be two subunits (i.e. a 64KD and a 57KD subunit) of the mycobacterial acetyl-CoA carboxylase enzyme, which had not completely dissociated despite boiling the mycobacterial samples prior to SDS-PAGE electrophoresis or the c.120KD proteins may represent a third mycobacterial carboxylase enzyme subunit.

To date, the only other cloned mycobacterial protein with a known biological function is the 46KD *M. leprae* protein described by Jacobs et al. (1986). These authors observed that this *M. leprae* protein was capable of complementing a mutation in the citrate synthase gene of *E. coli* and accordingly concluded that this 46KD *M. leprae* protein was *M. leprae* citrate synthase.
Humoral antibodies to *M. leprae* antigens are found in patients encompassing the entire leprosy spectrum, with lepromatous leprosy patients having the highest levels (Abe, 1973). Similarly, tuberculosis patients have humoral antibodies raised to *M. tuberculosis* antigens (Kaplan and Chase, 1980). Accordingly, although not associated with protective immunity (the cellular immune system is responsible for controlling the proliferation of *M. leprae* and *M. tuberculosis* bacilli and for conferring protection from disease on individuals), the humoral response to *M. leprae* and *M. tuberculosis* antigens has been considered by workers as a possible aid for the diagnosis of leprosy and tuberculosis.

A number of authors have reported the detection of common mycobacterial antigens and species-specific antigenic determinants of *M. leprae* and *M. tuberculosis* by using a variety of techniques: double diffusion precipitation (Stanford et al., 1976; Caldwell et al., 1979; Payne et al., 1982); by crossed-immunoelectrophoresis (Harboe et al., 1977; 1978; Kronvall et al., 1976; Closs et al., 1979); by immunoblotting (Chakrabarty et al., 1982; Klatser et al., 1984) and more recently with the use of monoclonal antibodies (Coates et al., 1981; Gillis and Buchanan, 1982; Ivanyi et al., 1983; Kolk et al., 1984; Schou et al., 1985; Young et al., 1985; Britton et al., 1985; Engers et al., 1985; 1986; Husson and Young, 1987; Buchanan et al., 1987).
With the aid of monoclonal antibodies, recombinant clones encoding epitopes of five *M. leprae* proteins (Young et al., 1985b) and five *M. tuberculosis* proteins (Young et al., 1985a; Husson and Young, 1987) have been isolated, encouraging these authors to speculate at the possibility of developing simple but specific diagnostic assays with these protein antigens, for screening populations to detect individuals producing antibodies to these antigens, thus making the early diagnosis of leprosy and tuberculosis feasible. It was further anticipated by these and other authors that the availability of mycobacterial antigens, produced by recombinant DNA technology, would result in the identification of antigenic epitopes responsible for T-cell proliferation (i.e. activation of the cell-mediated immune response) and their subsequent use in the production of (i) specific skin-test reagents for assessing the immunological status of patients (leprosy or tuberculosis patients) and their contacts or (ii) specific and effective vaccines.

In a series of experiments (sections 14 to 14.2), I attempted to determine whether the *M. leprae*-infection status of individuals could be elicited through the detection of antibodies to specific mycobacterial antigens in their antiserum. Antisera samples were subsequently tested for the presence of antibodies to (i) five *M. leprae* antigens (Young et al., 1985b) and (ii) six antigenic *M. bovis* BCG proteins.

The results of these experiments show that antibodies were detected to only one of the eleven mycobacterial antigens tested (the antigenic fusion protein expressed by gII/*M. bovis* BCG clone 532-9) and
moreover these antibodies were detected in the antiserum of only one lepromatous leprosy patient (LL serum No.6). Although none of the antisera samples tested, contained antibodies to any of the five \textit{M. leprae} antigens, I believe it would be prudent to screen many more antisera samples (for antibodies to these five antigens) before concluding that none of these \textit{M. leprae} antigens has a role to play in the early diagnosis of leprosy.

An unexpected result (from the screening experiments described in section 14.1) was the finding that the antigen expressed by gtlI/\textit{M. leprae} clone Y3184 was biotinylated. Young et al. (1985b) originally reported that this clone was detected with the aid of a mouse monoclonal antibody (i.e. ML-06 (Ivanyi et al., 1985) raised against a 12KD \textit{M. leprae} antigen) and biotin conjugated secondary antibodies. Recently however, Britton et al. (1987) reported their inability to detect the \textit{M. leprae} antigen expressed by clone Y3184, using a different monoclonal antibody (L22) but raised against the same 12KD \textit{M. leprae} antigen. These authors subsequently suggested that the clone Y3184 contained only a portion of the 12KD \textit{M. leprae} antigen, lacking the DNA encoding for the epitope, recognised by their own monoclonal antibody. From my results (and Britton et al's results), it is possible to speculate that: (1) the clone Y3184 was selected by Young et al. (1985b), solely for the ability of its expressed protein to react with the biotin conjugated secondary antibodies, used in the screening procedure (thus this clone is a false positive); (2) that this clone contains two partial genes, one encoding for a portion of the 12KD \textit{M. leprae} protein and the second encoding for a portion of a biotinylated
M. leprae protein or (3) the 12KD M. leprae protein is a biotinylated protein. The third possibility appears less likely, as from my results in section 11.2, I did not detect biotinylated proteins as small as 12KD.

In contrast to my own results, a number of authors have reported the detection of antibodies to the common 65KD mycobacterial antigen: Thole et al. (1985; 1987) and Levis et al. (1986) have reported (respectively) the presence of antibodies to this antigen in the antisera of (1) tuberculosis and (2) leprosy patients, thus raising the possibility of using the presence of antibodies to this antigen to diagnose these diseases. Thole et al. (1987) however, also reported the detection of antibodies to this antigen in as many as 30% of their control antisera samples (antisera from children with no history of mycobacterial infection) and these authors concluded that these individuals had been primed with antigens, cross-reactive with the common 65KD mycobacterial antigen. Young et al. (1987b) have recently reported their identification of bacteria not belonging to the genus Mycobacterium but containing antigens cross-reactive with the common 65KD mycobacterial antigen; results which imply that the common 65KD mycobacterial antigen is a common bacterial antigen and which could account for the results obtained by Thole et al (1987). Although these results appear to rule out the use of the complete 65KD mycobacterial antigen as a tool for diagnosing disease (leprosy or tuberculosis) the identification of species-specific epitopes of this antigen should overcome the problem of cross-reactivity and enable unique antigenic determinants of the 65KD antigen to be used as skin-test reagents. (The
identification of an \textit{M}.\textit{leprae}-specific epitope of the common 65KD mycobacterial antigen has recently been reported by Buchanan et al. 1987).

Following the results obtained in sections 14 to 14.2, a search was begun to identify mycobacterial antigens, capable of playing a role in the diagnosis of leprosy (section 15). A lack of sonicated \textit{M}.\textit{leprae} bacilli however, forced me to screen mycobacterial species other than \textit{M}.\textit{leprae}, to identify antigens able to cross-react with antibodies raised by leprosy patients. The species screened were \textit{M}.\textit{bovis} BCG, \textit{M}.\textit{phlei}, \textit{M}.\textit{vaccce}, \textit{M}.\textit{smegmatis} and \textit{"M}.\textit{lufu}" and from the photographs of the screening experiments (section 15), it can be seen that each antiserum tested, contained antibodies to many different mycobacterial antigens. There did not appear to be any obvious correlation between the antibody response to these mycobacterial antigens and the disease-status of the individual, although two antigens did show possibly significant differences between leprosy patients and both contacts and non-contacts (of leprosy patients): (1) a 30-40KD \textit{"M}.\textit{lufu}" antigen reacted with the antiserum from lepromatous leprosy patients only and (2) a c.24KD \textit{M}.\textit{bovis} BCG antigen reacted with contacts and non-contacts antiserum.

Determining a role for either or both of these antigens in the diagnosis of leprosy would require (1) soluble extracts of both \textit{M}.\textit{bovis} BCG and \textit{"M}.\textit{lufu}" to be screened with antiserum from many more leprosy patients, i.e. encompassing the complete leprosy spectrum and including newly diagnosed, pre-chemotherapy patients, to confirm (or disprove) the
relationship between the antibody response to these two antigens and disease status, which my preliminary results have implied and then (2) assessing the response of these two antigens to protease treatment, to determine whether these antigens are proteins or not (Britton et al., 1985; have reported the identification of a 30-40KD *M. leprae* antigen, reactive with antibodies in lepromatous leprosy antisera, which may be carbohydrate in nature). If the antigens are proteins, then recombinant clones, carrying the genes encoding for these antigens, can be recovered from the appropriate gtII gene library, i.e. gtII/*M. bovis* BCG or gtII/*M. lufen* and purified antigen could be prepared from these clones and used to test the level of corresponding antibody in the antisera of leprosy patients; contacts of leprosy patients and non-contacts.

All mycobacterial antigens which are identified (including my own *M. bovis* BCG antigens), should be tested for their ability to stimulate helper T-cells (regardless of their ability to stimulate humoral antibody production in leprosy or tuberculosis patients) as it is the cell-mediated immune response which is responsible for protecting individuals from these diseases. To date workers have identified a small number of mycobacterial antigens able to stimulate T-cells: the common 65KD mycobacterial antigen (Emmrich et al., 1986a; Oftung et al., 1987; Lamb et al., 1987; Gillis and Job, 1987; Thole et al., 1987; DeBruyn et al., 1987); an 18KD *M. leprae* antigen (Mustafa et al., 1986); a 36KD *M. leprae* antigen (Ottenhoff et al., 1986a) and a 19KD *M. tuberculosis* antigen (Oftung et al., 1987). The basic assumption behind any vaccine against leprosy is that induction of a state of immunological reactivity to *M. leprae* antigens will lead to protection
against infection and disease (Bloom and Mehra, 1984), therefore the
detection of antigens able to stimulate helper T-cells is imperative.
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The cloning of "Streptomyces lividans" to use the expression signals of genes from Mycobacterium bovis BCG was tested in vivo by using gene fusions. Random DNA fragments from M. bovis BCG were inserted into promoter-probe plasmids in Escherichia coli and in "S. lividans." Comparison with promoter activity detected with random DNA fragments from the respective hosts suggested that "S. lividans" efficiently utilizes a high proportion of mycobacterial promoters, whereas a smaller fraction are expressed, and expressed more weakly, in E. coli. M. bovis BCG DNA fragments were also inserted into the specially constructed translational fusion vector (pJ688) in "S. lividans." pJ688 contains the kanamycin phosphotransferase gene (neo) from transposition Tn5, truncated at its amino terminus, as the indicator. The results suggested that "S. lividans" uses M. bovis BCG translational signals almost as efficiently as its own signals. Moreover, several hybrid proteins with an M. bovis BCG-derived amino terminus seemed to be reasonably stable in "S. lividans." These experiments indicate that "S. lividans" may be a suitable host for the expression of Mycobacterium leprae and Mycobacterium tuberculosis genes from their own signals. This is a precondition for the expression of entire biosynthetic pathways, which could be valuable in the production of diagnostic and therapeutic agents. The vectors may also have wider applications for the analysis of gene expression in Streptomyces.

Tuberculosis and leprosy are still among the world's major infectious diseases. They are caused by the slow-growing Mycobacterium tuberculosis and Mycobacterium leprae, both of which grow intracellularly in the host (obligately in the case of M. leprae), making chemotherapy difficult. The slow growth rates of both pathogens (doubling times in vivo of 15 to 20 h and 12 to 20 days, respectively) and the failure of M. leprae to grow in vitro have constrained the study of these bacteria. Gene cloning provides a way of producing mycobacterial proteins in more convenient organisms. The availability of such gene products should help in the refinement of diagnostic agents and possibly the development of vaccines, as well as in screening for chemotherapeutic agents targeted on specific mycobacterial enzymes. The latter becomes increasingly important with the emergence of resistant strains.

Recently, considerable progress has been made in the expression of mycobacterial genes in Streptomyces. Experiments in which M. leprae DNA was cloned in E. coli cosmid vectors suggested that mycobacterial genes could, at best, be expressed only weakly from their own signals in this host (8), a conclusion reinforced by cloning M. bovis BCG DNA in lambda EMBL 3 (28). By the use of expression vectors, clones were isolated which complemented E. coli auxotrophic mutations (8) or which produced polypeptides reacting with monoclonal antibodies specific for M. leprae and M. tuberculosis (28, 34, 35). A particular Mycobacterium fortuitum plasmid gene was shown to be expressed from its own promoter (21).

The use of an alternative host which would express mycobacterial genes without requiring an expression vector not only would accelerate the search for further mycobacterial genes, but might also make it possible to obtain expression of multigene pathways. These pathways would include that responsible for the biosynthesis of the M. leprae-specific phenolic glycolipid which is an immunodominant antigen (15, 33). This objective should be aided by the general tendency of genes for biosynthetic pathways to be clustered in bacterial genomes. Nonpathogenic relatives of pathogenic Mycobacterium strains are the most likely hosts to express M. tuberculosis or M. leprae genes, but cloning procedures for mycobacteria have not yet been developed. Streptomycetes are nonpathogenic gram-positive bacteria which belong, in common with the mycobacteria, to the order Actinomycetales, even though the G+C content of Streptomyces spp. DNA (about 73% [9]) is higher than that of the mycobacteria (56 to 65% [8]). Efficient cloning and in vivo genetic procedures are available for "Streptomyces lividans" (13), which is a convenient, nonrestricting host. Moreover, existing evidence suggests that streptomycetes are quite versatile in their ability to recognize heterologous procaryotic promoters (2, 16). (Streptomyces promoters are themselves heterogeneous in sequence [12], and multiple forms of RNA polymerase are at least partly responsible for the transcription of different promoter classes [32].)

To investigate the general feasibility of cloning and expressing Mycobacterium genes in "S. lividans," we have explored the ability of transcription and translation signals in DNA from the nonpathogenic M. bovis BCG strain to activate indicator genes in suitable plasmid vectors. The level of in vivo expression of the indicator gene was used to survey the strength of the cloned signals, and the result was assessed by comparing the level of expression achieved with random heterologous (in our case BCG) or homologous ("S. lividans") DNA fragments. A feature of this approach is that, with a single assay, the efficiency of a random sample of all promoters and translational signals can be examined in vivo without prior knowledge of gene functions. The results are of course influenced by other factors, notably the stability of the hybrid mRNAs and, in translational fusions, of the hybrid proteins. However, since we are primarily interested in the production of mycobacterial proteins in "S. lividans"...
rather than in a precise assessment of the contributions of different factors to overall gene expression, this could be an advantage. We have used high-copy-number Streptomyces plasmid vectors which might be expected to lead to increased production of proteins from some of the cloned genes through gene amplification. The neo gene from the E. coli transposon Tn5 was the indicator gene. This gene codes for an aminoglycoside phosphotransferase (NPT II) that confers resistance to kanamycin (or neomycin) on "S. lividans." The level of resistance varies widely, depending on the amount of NPT II produced, and can therefore be used as a convenient indicator of the level of gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. "S. lividans" 66 strain TK64 (str-6 pro-6, plasmid-free) (14) was the host for the transformation with pIJ424 and its derivatives (31a). E. coli HB101 (6) was the host for transformation with pKK223-2 (P-L Biochemicals) (7) and its derivatives. M. bovis BCG (Glaxo) was obtained from J. Morris (Central Veterinary Laboratory, Weybridge, U.K.).

Culture media, antibiotic selection, and transformation procedures. Techniques for streptomycetes were as described in detail by Hopwood et al. (13). "S. lividans" was grown in liquid YEME medium with 34% sucrose and 0.5% glycite for protoplasting and for the preparation of protein extracts. Tryptone soya broth (Oxoid, CM 129) was used to grow mycelium for plasmid isolation. R2YE agar was used for the regeneration of protoplasts and for the preparation of spore suspensions, and MM agar was used for the selection of kanamycin-resistant strains. All media for streptomycetes were supplemented with proline to allow growth of strain M. bovis BCG, as well as 4 g of asparagine and 0.2 g of Tween 80 with antibiotics, as appropriate. Transformation of E. coli with plasmid DNA was done by the method of Kushner (20). Bacterial strains and plasmids. "S. lividans" 66 strain TK64 was grown in Santon medium, pH 7.0,

Preparation of cell extracts. Soluble protein extracts from "S. lividans" were prepared from mycelium washed twice with the sample buffer of Reiss et al. (25). The cells were broken by sonication in sample buffer, and the lysate was spun for 10 min at 4°C in an Eppendorf centrifuge to remove cell debris. Samples were stored at 70°C.

Polycrylamide gel electrophoresis. Polycrylamide (10%) gels were prepared, run, and stained with Coomassie blue by procedure 53 of Silhavy et al. (27). For nondenaturing gels, sodium dodecyl sulfate (SDS) was omitted from all the buffers and the samples were not boiled. About 50 µg of protein was loaded into each gel slot.

Detection of kanamycin phosphotransferase activity in native polycrylamide gels. The procedure used to detect kanamycin phosphotransferase activity was that of Reiss et al. (25) with the following specifications: 100 µCi of [γ-32P]ATP (3,000 Ci/mmol) was used for each agarose gel (150 by 200 by 1 mm). The PS1 phosphocellulose paper was washed three times each for 1 h with 50 mM sodium phosphate buffer, pH 7.4, and twice with cold water before being dried and exposed to X-ray film.

Detection of kanamycin phosphotransferase by Western blotting. Proteins separated on SDS-polycrylamide gels were transferred electrophoretically to nitrocellulose (Schleicher & Schuell BAS5) (29) with 25 mM sodium phosphate buffer, pH 6.5, at room temperature with a Bio-Rad Trans-Blot Cell (ca. 10 V and 0.2 A for 14 h). The filter was washed and reacted sequentially with 1:1,000 diluted rabbit anti-NPT II serum (kindly gift from J. Davies, Biogen S.A., Geneva) and 1:500 diluted goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (kindly provided by R. Casey, John Innes Institute), and then stained by the procedure of Blake et al. (5).

RESULTS

Cloning of M. bovis BCG DNA fragments into the Streptomyces promoter-probe plasmid pIJ424. pIJ424 (31a) carries the replication region of the high-copy-number, wide-host-range Streptomyces plasmid pIJ101, the thiostrepton resistance gene (srr) for selection, the promoterless kanamycin resistance determinant (neo), and a transcriptional terminator (ter) from the E. coli phage F (Fig. 1). Between the unique BglII cloning site (which is downstream from the stop codon for neo) and the ATG start codon of the plasmid. When a DNA fragment with promoter activity is inserted in the correct orientation into the BglII site. The level of kanamycin resistance conferred on "S. lividans" varies between about 2 and more than 500 µg/ml, depending on the strength of the cloned promoter.

To generate a BCG gene library in "S. lividans," 5 µg of BCG DNA was digested with BglII and ligated to 1 µg of pIJ424 digested with BglII and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). After transformation of "S. lividans" protoplasts, about 150,000 thiostrepton-resistant transformants were selected and used for growth on kanamycin (5 µg/ml). An estimated 50,000 of these colonies were resistant to kanamycin. Analysis by agarose gel electrophoresis of plasmid DNA from 80 such colonies showed that all clones contained plasmids larger than the vector. Most inserts were between 0.5 and 10 kilobases (kb) in size (average, 5 kb), reflecting the size distribution of the BglII-cut BCG DNA, and thus probably represented a random sample of BCG DNA. There was no indication of the multiple occurrence of particular DNA fragments. This experiment suggested that BCG DNA contains a large number of promoters active in "S. lividans."
Plasmid DNA from 12 kanamycin-resistant clones was purified and reintroduced into "S. lividans" protoplasts. All transformants were kanamycin resistant, confirming that in each case the resistance was plasmid-borne. The 12 plasmids were digested with BglII and religated, regenerating the original pIJ424 vector. After introduction into "S. lividans," thiostrepton-resistant transformants were recovered and found to be kanamycin sensitive. This confirmed that the kanamycin resistance of the clones was due to the cloned DNA fragments and not a consequence of mutations or rearrangements of the vector plasmid.

To eliminate the possibility that DNA from a chance contaminant had been cloned, DNA was isolated from a second BCG culture. After digestion with BglII, the sample of DNA used for the cloning experiment and the new sample gave the same characteristic banding patterns on an agarose gel, confirming their identity.

The unlikely possibility that residual chromosomal DNA from "S. lividans" in the vector DNA preparation might have been the source of the observed promoter activity was excluded for one clone by using 32P-labeled (nick-translated) plasmid DNA from a highly kanamycin-resistant clone as the probe in a Southern hybridization experiment against BglII-digested DNA from BCG and "S. lividans." The BCG DNA gave a single positive signal in the position corresponding to the size of the cloned fragment, with no hybridization to the "S. lividans" DNA.

The above experiments, coupled with the finding that the clones contained plasmids of many different sizes, established that BCG DNA can be cloned with high efficiency in "S. lividans" and that many BCG DNA fragments show promoter activity in this host. When "S. lividans" was transformed with undigested pIJ424, fewer than 1% of the thiostrepton-resistant colonies could grow after replication to kanamycin (5 \( \mu \)g/ml). Usually only part of a colony (possibly only a single transferred spore) was kanamycin resistant. The kanamycin-resistant colonies obtained with cloned BCG DNA, on the other hand, were always completely resistant and replicated as small patches. Plasmid DNA from 12 spontaneously kanamycin-resistant mutants was reintroduced into "S. lividans." Kanamycin-resistant colonies were recovered, proving that the mutations were plasmid-borne. Two of these plasmids were smaller than pIJ424 and stable, but 10 were larger, of various size, and unstable, regenerating vector-size molecules which no longer conferred kanamycin resistance.

**Quantitative comparison of promoter activity in "S. lividans" of DNA fragments from BCG or "S. lividans."**

Randomly selected thiostrepton-resistant colonies from the BCG library were tested for their level of kanamycin resistance and for possession of inserted DNA. Fifty percent were found to contain vector plasmids with no detectable insert; all of these were kanamycin sensitive. Of 270 clones which contained BCG DNA, a surprisingly high 69% were resistant to at least 5 \( \mu \)g of kanamycin per ml. Since about 50,000 kanamycin-resistant clones were recovered (see above), an estimated 72,000 BCG clones were obtained in this experiment.

These results can be compared with those obtained with a library of "S. lividans" fragments cloned in the same manner. A similar proportion lacked inserts, and again these were all kanamycin sensitive. In this case, of 190 clones containing "S. lividans" DNA, 78% were resistant to at least 5 \( \mu \)g of kanamycin per ml. The similarity of this figure to that

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**FIG. 1.** Restriction maps of the Streptomyces promoter-probe plasmid pIJ424 and the translational fusion vector pIJ688. The two plasmids differ at the amino terminus of the neo gene, and pIJ688 lacks the BamHI site shown in parentheses. (The derivation of pIJ688 from pIJ424 is explained in Fig. 4.) The sequence at the amino terminus of the neo gene in pIJ424 is from Beck et al. (1) and was confirmed in a derivative of pIJ424 by Ward et al. (31a). The sequence of pIJ688 is deduced from the results of Reiss et al. (25) for pKM109-90 and Ward et al. (31a) for pIJ486. neo, Kanamycin phosphotransferase gene from Tn5; RBS, ribosome binding site. Stop codons and the ATG start codon for the neo gene are underlined. Asterisks indicate the cloning sites.

Plasmid DNA from 12 kanamycin-resistant clones was purified and reintroduced into "S. lividans" protoplasts. All transformants were kanamycin resistant, confirming that in each case the resistance was plasmid-borne. The 12 plasmids were digested with BglII and religated, regenerating the original pIJ424 vector. After introduction into "S. lividans," thiostrepton-resistant transformants were recovered and found to be kanamycin sensitive. This confirmed that the kanamycin resistance of the clones was due to the cloned DNA fragments and not a consequence of mutations or rearrangements of the vector plasmid.

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The unlikely possibility that residual chromosomal DNA from "S. lividans" in the vector DNA preparation might have been the source of the observed promoter activity was excluded for one clone by using 32P-labeled (nick-translated) plasmid DNA from a highly kanamycin-resistant clone as the probe in a Southern hybridization experiment against BglII-digested DNA from BCG and "S. lividans." The BCG DNA gave a single positive signal in the position corresponding to the size of the cloned fragment, with no hybridization to the "S. lividans" DNA.

The above experiments, coupled with the finding that the clones contained plasmids of many different sizes, established that BCG DNA can be cloned with high efficiency in "S. lividans" and that many BCG DNA fragments show promoter activity in this host.
**FIG. 2.** (A) Kanamycin resistance levels of pIJ424 clones (transcriptional fusions) in "S. lividans" TK64 containing BgllII fragments of BCG DNA or "S. lividans" DNA. (B) Chloramphenicol resistance levels of pKK232-8 clones (transcriptional fusions) in *E. coli* HB101 containing BamHI fragments of BCG or *E. coli* DNA. The heights of the bars represent the percentages of clones which grew on medium with the indicated concentration of antibiotic but not with the next higher concentration.

**FIG. 3.** Simplified restriction map of the *E. coli* promoter-probe plasmid pKK232-8 (7). The main features are the ampicillin resistance determinant (*amp*) for selection and the promoterless chloramphenicol resistance gene (*cat*) which codes for chloramphenicol acetyltransferase (CAT). *T*₁ and *T*₂ are transcriptional terminators from the *rrnB* gene. The sequence of the amino-terminal region of the *cat* gene is combined from data published by Brosius (7) and Vieira and Messing (31). The ATG start codon for the *cat* gene and stop codons (present in all three reading frames) are underlined. RBS, Ribosome binding site.

Obtained with BCG DNA (69%) suggests that the majority of BCG promoters are recognized in "S. lividans." The size distribution of the inserts was approximately the same for both sets of clones.

The relative strengths (in "S. lividans") of the BCG and "S. lividans" promoters can be assessed by comparing the level of kanamycin resistance of individual clones. Figure 2A shows that fewer of the BCG clones exhibited high-level resistance to kanamycin, indicating that the level of expression from the BCG promoters is on average somewhat lower than that obtained from the homologous "S. lividans" DNA fragments.

The isolation of kanamycin-resistant clones at a frequency of more than 50% is remarkable and raises the question whether the detection system was too sensitive, possibly because of the high copy number (ca. 100 per chromosome) of pIJ424, leading to initiation of transcription at DNA sequences which are not used as promoters under normal circumstances. Kanamycin resistance in more than 50% of the clones would be expected, however, if promoters are frequently in a "back to back" arrangement in the genome and if termination of transcription is often incomplete, as is the case in *E. coli* (26).

"S. lividans" TK64 containing pIJ424 is slightly more resistant to kanamycin than the plasmid-free strain; the difference can be detected on kanamycin gradient plates (31a). It is not known whether this slight kanamycin resistance is due to incomplete termination at the *fd* terminator or to initiation of RNA synthesis at a weak promoter downstream of the termination point (R. Gentz, personal communication). Seventy clones sensitive to 5 μg of kanamycin per
ml were tested on gradient plates. Six of these clones were more sensitive to kanamycin than was strain TK64 containing pIJ424 and probably contained transcriptional terminators.

Promoter activity of BCG DNA in E. coli HB101. To clarify the expression of mycobacterial genes in E. coli and to provide a comparison for the "S. lividans" result, a similar experiment was performed with the E. coli promoter-probe plasmid pKK232-8 (Fig. 3) (7) and BamHI-generated fragments of DNA from BCG or E. coli HB101.

With BCG DNA, 1,043 ampicillin-resistant transformants were obtained; 170 were found to be resistant to at least 5 µg of chloramphenicol per ml, i.e., showed promoter activity, and 873 were sensitive. Plasmids from samples of chloramphenicol-resistant and -sensitive colonies were analyzed; all the resistant colonies contained plasmids with inserts, as did 75% of the sensitive colonies. (The average insert size was 5 kb.) Thus, 21% [100 x 170 / (170 + 873 x 0.75)] of the total clones with inserts showed promoter activity.

With E. coli DNA, 115 of 461 ampicillin-resistant transformants were resistant to at least 5 µg of chloramphenicol per ml, and 346 were sensitive. All resistant clones had inserts, as did 50% of the sensitive. (The average insert size was 7.5 kb.) Thus, 40% [100 x 114 / (115 + 346 x 0.5)] of the clones with inserts showed promoter activity. (The fact that this value is much lower than that obtained with homologous DNA in the "S. lividans" promoter-probe experiment [78%] may not be biologically significant; it could simply indicate that pKK232-8 is less sensitive in detecting the presence of very weak promoters.)

The relative strengths of the homologous and heterologous promoters in E. coli were assessed by testing the levels of chloramphenicol resistance of individual clones (Fig. 2B). A comparison with the "S. lividans" results (Fig. 2A) suggests that most BCG promoters compare better with homologous promoters in "S. lividans" than in E. coli, in which only a small minority of BCG promoters were strongly active.

Construction of pIJ688, a translational fusion vector for streptomycetes. Having established that many BCG promoters are active in "S. lividans," we set out to determine whether BCG translational start signals also function in this organism. For this purpose we replaced the promoterless neo gene in pIJ424 with the truncated neo gene from pKM109-90 (24), which lacks the ribosome binding site and the codons for the first four amino acids, including the ATG start codon (Fig. 1). The construction of pIJ688 is explained in Fig. 4. Kanamycin resistance is expected to occur only when a DNA fragment with a promoter and a translational start signal is fused in the right orientation and reading frame to the amino terminus of the truncated neo gene. (The only unique BamHI site of pIJ688 can be used for making translational gene fusions, because there is an in-frame TAG stop codon in the XbaI recognition sequence [Fig. 1].)

Quantitative comparison of kanamycin resistance levels obtained in "S. lividans" with DNA fragments from BCG or "S. lividans" cloned into the translational fusion vector pIJ688. BamHI-digested BCG and "S. lividans" DNA samples were ligated to BamHI-digested and calf intestinal alkaline phosphatase-treated pIJ688 DNA and introduced into "S. lividans" by transformation. Samples of the resulting transformants (154 for the experiment with BCG DNA and 149 for that with "S. lividans" DNA) were analyzed by gel electrophoresis: 106 and 80 of the colonies, respectively, had detectable inserts. Eight percent (9 of 106) and 13% (10 of 80) of the clones with detectable inserts from BCG and "S. lividans" conferred resistance to at least 1.5 µg of kanamycin per ml (pIJ688, unlike the promoter-probe plasmid pIJ424, does not increase the kanamycin resistance of S. lividans). Plasmids from eight kanamycin-resistant clones containing BCG DNA inserts were isolated and reintroduced into "S. lividans," where again kanamycin resistance was
observed, showing that the resistance was plasmid-borne. The eight plasmids were also digested with BamHI (to remove the inserts) and religated to regenerate pIJ688. Kanamycin-sensitive "S. lividans" transformants were recovered, showing that resistance depended on the cloned BCG DNA.

The maximal expected frequency of active fusions would be one in six (16.6%), assuming that all DNA is coding, that the DNA must be fused in the correct orientation and reading frame, and that every hybrid protein is enzymatically active or degraded to an active form intracellularly. Taking into account the results of the promoter-probing experiment (Fig. 2A), that about 25% of all the clones were kanamycin sensitive and therefore had no detectable promoter activity, one would expect up to 12.5% (75% of 16.6%) of all the clones to show kanamycin resistance. The actual values were close to this figure, suggesting that (nearly) all fusions in the correct reading frame give rise to kanamycin resistance. This interpretation must of course be treated with caution in view of the many assumptions implicit in the calculation. (It should be noted that the placement of the BamHI site with respect to the reading frame in pIJ688 [Fig. 1] favors gene fusion in the correct reading frame, because both "S. lividans" and BCG have a high G+C content and thus mostly G or C in the third codon position.)

Having established that kanamycin-resistant colonies carried plasmids with inserts, larger samples of colonies growing on 1.5 μg of kanamycin per ml (316 and 318 clones, respectively, for the experiments with BCG and "S. lividans" DNA) were tested on higher levels of kanamycin (Fig. 5). The average resistance level was lower than for the transcriptional fusions in pIJ424. This agrees with the expectation that many of the hybrid proteins would have a lower specific kanamycin phosphotransferase activity than the original protein. There was little difference in the number of resistant clones at different kanamycin concentrations obtained with BCG or "S. lividans" inserts except for the two lowest concentrations. This suggests that BCG promoters and translational start signals in their natural relative config-

FIG. 5. Kanamycin resistance levels of pIJ688 clones (translational fusion) in "S. lividans" TK64 with BamHI fragments of BCG or "S. lividans" DNA. Bars indicate the percentages of clones that grew on medium with the indicated concentration of drug but not with the next higher concentration.

FIG. 6. Detection of kanamycin phosphotransferase activity in lysates of "S. lividans" TK64 on a nondenaturing polyacrylamide gel. Lane d, pIJ688 without insert; lanes h through k, p, and q, transcriptional fusions with pIJ424 as vector; lanes a, b, c, e through g, and l through o, translational fusions with pIJ688; lanes B, D, and Q, Coomassie-stained gels with the same extracts as in lanes b, d, and q. The heavy arrow indicates the position of NPT II as produced from the transcriptional fusions. The light arrows indicate the position of the abundant fusion protein in lane B and its activity stain in lane b. All the clones except in lanes d and i contained BCG inserts. Lane i contains an "S. lividans" insert.
FIG. 7. Detection of kanamycin phosphotransferase in lysates from "S. lividans" TK64 with various plasmids, on an SDS-polyacrylamide gel. The gel was blotted to nitrocellulose and the filter was first reacted with rabbit anti-NPT II serum and then with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase. Staining was with indoxyl phosphate. The letters for the lanes correspond to those in Fig. 6. (A) Stained nitrocellulose filter. (B) NPT II-specific bands are drawn selectively. The dotted line indicates the position of a 27.5-kilodalton protein like the authentic NPT II. There are many nonspecific bands in control lanes 7 and 8. Lanes 1 through 4, Translational fusions of BCG DNA in pIJ688; lane 7, TK64 with pIJ424; lane 8, TK64 with pIJ688; lanes 5, 6, 9, and 10, transcriptional fusions with pIJ424. The bands migrated from top to bottom. The numbers to the right indicate the positions of size markers (in kilodaltons).

Detection of kanamycin phosphotransferase activity in non-denaturing polyacrylamide gels (activity blot). Since the translational fusion vector (pIJ688) had not been used before, we needed to demonstrate that fused proteins were indeed generated from the clones in "S. lividans." We also wished to confirm that the neomycin phosphotransferase from the transcriptional fusions was identical in size with the authentic enzyme, as predicted from the sequence (Fig. 1). Soluble extracts from highly kanamycin-resistant clones were subjected to electrophoresis on non-denaturing polyacrylamide gels. Phosphotransferase activity was located by the method of Reiss et al. (25). The results (Fig. 6) show, for all transcriptional (pIJ424) fusions, a single band migrating at the same speed as authentic neomycin phosphotransferase (heavy arrow). In contrast, each translational (pIJ688) fusion showed multiple bands, all migrating more slowly than the authentic enzyme. Several of the lysates showed extra bands on the gels stained with Coomassie blue, and these were approximately in the region where the kanamycin phosphotransferase activity was detected. In Fig. 6, particularly strong bands from a transcriptional fusion (heavy arrow, lanes q and Q) and a translation fusion (light arrows, lanes b and B) are shown. Lane D in Fig. 6 shows the banding pattern after Coomassie blue staining obtained with a lysate of "S. lividans" containing pIJ688 with no insert. These strong bands indicate that the neomycin phosphotransferase, which originates from the E. coli Tn5, and at least some of its hybrid derivatives can be highly expressed and stably accumulated in "S. lividans."

Detection of kanamycin phosphotransferase in Western blots with specific antibodies. Since the activity blot (Fig. 6) only revealed the positions of enzymatically active proteins, some of the proteins might have been subject to extensive degradation to inactive polypeptides. To clarify this point, extracts of four pIJ424 and four pIJ688 clones containing BCG inserts were subjected to electrophoresis on a denaturing polyacrylamide gel and then blotted to a nitrocellulose filter. The filter was first reacted with rabbit anti-NPT II serum and then with goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase. The developed filter (Fig. 7) showed multiple bands in the control lanes, which may have been due to cross-reacting antibodies in the goat or rabbit serum or to renatured phosphatases from the "S. lividans" extracts. The clone-specific bands could, however, easily be identified (and are drawn in Fig. 7B). All the pIJ424 clones gave a single band (the lower part of the gel, which was devoid of specific bands, is not shown in Fig. 7), indicating good stability of this E. coli protein in "S. lividans." The clones from pIJ688 gave the same number of multiple bands as in the activity blot, but here the largest band was always the strongest, indicating that the primary fusion protein was accumulated preferentially and that possibly all the tested hybrid NPT II proteins and their degradation products are enzymatically active.

DISCUSSION

We have shown that DNA from M. bovis BCG can be cloned efficiently in "S. lividans" by using multicopy plasmid vectors. Random BCG DNA fragments seem to have a better chance of being transcribed in S. lividans than in E. coli. Although it is possible that initiation of transcription for a fraction of these fragments might occur at sites which are not promoters in M. bovis, we believe that the majority of the active fragments carry true promoters. Our results are consistent with earlier findings (8, 28), which showed that most mycobacterial genes fail to be expressed in E. coli, and also with the results of Bibb and Cohen (2) and Jaurin and Cohen (16, 17), who showed that E. coli promoters are transcribed when cloned into Streptomyces promoter-probe plasmids, even though Streptomyces DNA contains few sequences which act as promoters in E. coli. One should not ignore the finding that some BCG DNA fragments showed promoter activity in E. coli, a result compatible with those already reported (21, 28).
The newly constructed translation fusion vector (pIJ688) was shown to be useful for the generation of enzymatically active (NPT II) hybrid proteins in "S. lividans." Analysis of the kanamycin resistance of pIJ688 clones indicated that BCG transcriptional and translational signals compare well with those in the "S. lividans" genome. Encouraging for our goal (the cloning and expression of specific M. leprae and M. tuberculosis genes) is the fact that we could detect clone-specific protein bands in total soluble "S. lividans" extracts on Coomassie-stained gels. In some cases these bands could be aligned with kanamycin phosphotransferase-specific bands seen in activity blots or Western blots. This proved that they were proteins expressed from BCG signals rather than chromosomal or plasmid genes which might have been induced indirectly by the presence of the hybrid plasmids, as has been described for E. coli by Goff and Goldberg (11), or (unexpectedly) hybrid proteins with a vector-specific amino terminus and a carboxy terminus from the cloned fragment.

The data on gene fusions give only general information about uncharacterized genes and may not hold for each individual gene which might be of (medical) interest. It is possible, however, that Mycobacterium genes which are highly expressed in "S. lividans" are also highly expressed in their original host and may be good candidates for genes coding for important antigens. It is also possible that pathogenic mycobacteria need to induce specific genes on entering their mammalian host. A likely stimulus is an increase in temperature, as has been shown for parasitic protozoa (30). The heat shock response is a very general phenomenon in pro- and eucaryotes, and temperature induction of mycobacterial genes cloned in "S. lividans" might occur.

Other applications, besides the production of antigens, that could profit from a convenient and efficient cloning system in a host capable of expressing mycobacterial genes are the search for exported proteins and for gene clusters which code for biosynthetic pathways. Actinomycetes are known for their ability to produce hundreds of compounds (secondary metabolites), many of which have antibiotic activity, and it has recently been possible to clone in Streptomyces species an entire pathway for the biosynthesis of an antibiotic (23). Although no antibiotics have been found in mycobacteria, extracellular glycolipids are common. A further application could be the cloning of resistance determinants from drug-resistant clinical isolates which might be expressed in Streptomyces species. The products of these cloned genes could be used to investigate drug resistance mechanisms. This is important because the slow growth of mycobacterial pathogens hinders normal resistance testing.

The neo gene from Tn5 has proved to be a very useful indicator for both transcriptional and translational fusions with high-copy-number Streptomyces plasmid vectors. Our results from the quantitative analysis of the frequency and resistance levels obtained with random DNA fragments from total "S. lividans" or BCG DNA suggest that even the weakest promoters and most in-frame translational fusions result in a detectable increase in kanamycin resistance. The level of resistance varies over a wide range and, under controlled conditions, can be used as a measure of relative signal strength. These features make pIJ424, pIJ688, and similar gene fusion vectors (31a; Kieser, unpublished) potentially very useful for the study of gene expression in Streptomyces spp. The very high frequency (>50%) of random kanamycin-resistant clones also makes it possible to use kanamycin resistance to select plasmids which carry cloned DNA (D. A. Hopwood, M. J. Bibb, K. F. Chater and T. Kieser, Methods Enzymol., in press). With randomly sheared or partially digested target DNA, it might even be possible to achieve nearly complete representation of a whole genome with the benefit of positive selection. This would replace the use of dephosphorylated vectors, which yield fewer recombinant clones.

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EXPRESSION OF M. BOVIS BCG DNA IN "S. LIVIDANS"


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Expression of biotinylated proteins in mycobacterial gene libraries

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1. SUMMARY

A gene library of Mycobacterium bovis DNA in lambda gt11 was screened for the expression of M. bovis proteins using monoclonal antibodies and the streptavidin-biotinylated peroxidase detection system. Analysis of several phage that gave positive signals revealed that identical results could be obtained in the absence of specific antibody. These recombinants appear to encode biotinylated M. bovis proteins. A similar result was obtained with independently constructed libraries of M. bovis BCG and M. leprae.

2. INTRODUCTION

The lambda phage vector gt11 [1] is widely used for gene libraries when antibody screening is to be used to identify recombinants which carry and express the required genes. One very sensitive system for detecting bound antibody involves the use of a biotin-labelled second antibody and the high-affinity multivalent biotin-binding protein streptavidin [2]. Streptavidin is available commercially as a complex with biotinylated horseradish peroxidase, which is detected using a chromogenic substrate.

Recombinant clones from mycobacterial gene libraries which produced strong positive signals with the streptavidin system were isolated and characterised. On further testing it was shown that these signals were obtained independently of the binding of primary antibody, and that these false positive results appear to be due to the expression in Escherichia coli of biotinylated mycobacterial proteins. Although the problems of using streptavidin-based probes are well-known, this particular effect does not appear to be documented in the existing literature; the purpose of this communication is therefore to draw attention to this problem.

3. MATERIALS AND METHODS

3.1. Bacterial strains and other materials

Field isolates of M. bovis and the Glaxo strain of M. bovis BCG were provided by Dr. J. Morris, Central Veterinary Laboratory, Weybridge, Surrey. E. coli strains Y1088, Y1089 and Y1090 [1], lambda gt11 arms and ‘Gigapack plus’ in vitro packaging kit, were obtained from NBL enzymes Ltd. Mouse monoclonal antibodies specific for M.
bovis antigens were provided by A. Nolan, Central Veterinary Laboratory, Weybridge, Surrey. Nitrocellulose filters were Schleicher and Schuell products. IPTG (isopropyl β-D-thiogalactopyranoside), biotin, unlabelled streptavidin and the peroxidase substrate 4-chloro-1-naphthol were from Sigma. Biotinylated anti-mouse antibody and streptavidin-biotinylated peroxidase complex were from Amersham International plc. Restriction endonucleases and other enzymes were obtained commercially and used according to the manufacturers’ directions or as described by Maniatis et al. [3].

3.2. Construction and screening of gene libraries

Mycobacterial DNA was prepared as described [4] and partially digested with Sau3A to yield fragments of 2–9 kb. These were ligated with λgt11, using EcoRI linkers [5], and packaged in vitro. The gene library was amplified by infection of E. coli Y1088. The amplified library was screened after infection of E. coli Y1090 and transfer of phage proteins to nitrocellulose filters [1]. The filters were blocked with Tris-buffered saline-Tween-20 (TBST) containing 20% foetal calf serum, probed with mouse monoclonal antibodies and then incubated with biotinylated anti-mouse IgG and streptavidin-biotinylated peroxidase complex. This complex and the colour development substrate were used as directed by the manufacturers. The harvesting of bacteriophage from plate lysates and the extraction of DNA was accomplished by a scaled-down version of the protocol in Maniatis et al. [3].

4. RESULTS AND DISCUSSION

The M. bovis gene library was initially probed with MBS43, a monoclonal antibody specific for a M. bovis glycoprotein of approximately 26 kDa (A. Nolan, unpublished). Three isolates, A1.6, B1.2 and I1.4, were found to give positive signals in the absence of IPTG. This suggests that these genes are transcribed from their own promoters, rather than from the lac promoter of the vector. Kieser et al. [4] have shown that a minority of mycobacterial promoters are active in E. coli. Isolates A1.6 and B1.2 were found to harbour inserts of 3.1 kb, while I1.4 contained a slightly larger DNA fragment.

Plaques generated by these phages were subsequently probed with a second monoclonal antibody CMA 134.1, which reacts with a M. bovis protein of about 18 kDa, and which does not cross-react with the antigen recognised by MBS43 (A. Nolan, personal communication). Surprisingly, all three isolates gave positive results with the second monoclonal antibody. As it seemed unlikely that each of these recombinant phage would encode two antigenically distinct proteins, these clones were investigated further.

The filters carrying bound plaque proteins gave a negative result when incubated with the peroxidase substrate alone. On the other hand, a strong positive signal was obtained by the use of the streptavidin complex in the absence of antibody. This indicates that the effect is not due to direct interaction with the peroxidase substrate, nor to non-specific binding of the secondary antibody but is caused by the streptavidin complex being bound to proteins encoded by the recombinant phage.

This was tested by attempting to block the binding reaction with unlabelled streptavidin. One half of a filter was treated with streptavidin (2.5 μg/ml), the remainder being kept in TBST. After thorough washing, the remaining biotin-binding sites on the bound streptavidin were saturated by incubation with biotin (1 μg/ml). Both filter halves were then incubated with streptavidin-peroxidase complex. After the standard colour development procedure, only the untreated filter portions gave positive signals; those portions pre-treated as described above gave no reaction. This blocking effect of unlabelled streptavidin was eliminated if the streptavidin was saturated with biotin prior to use. This strongly suggests that the false positive reactions are due to the expression of biotinylated proteins.

Further characterisation was carried out by Western blotting. A temperature-sensitive lysogen was obtained by infection of E. coli Y1089 with the recombinant phage A1.6. A lysate was prepared as described by Huynh et al. [1], except that IPTG was omitted, and subjected to SDS-PAGE;
the proteins were electrophoretically transferred to a nitrocellulose filter. Incubation with streptavidin-biotinylated peroxidase complex and colour development solution (with no antibody treatment) disclosed up to 7 discrete bands with molecular weights between 53 and 91 kDa (Fig. 1, track A). Two of these bands (69 and 81 kDa) corresponded in size with two streptavidin-reactive bands in a sonicated extract of \textit{M. bovis} (track B). No reactive proteins were seen in a lysate of \textit{E. coli} Y1089. All of the streptavidin-reactive bands detected in the lysogen were too small to be fusions with the vector-encoded \(\beta\)-galactosidase. The additional bands may indicate that in this host a larger precursor is produced which is then sequentially (and perhaps aberrantly) processed to give the range of reactive bands observed, two of which correspond in size to the native protein in \textit{M. bovis}. Analysis of the other recombinant phages B1.2 and I1.4 gave results identical to those above. A similar effect has been observed with an independently constructed \(\lambda\) gt11 gene library of \textit{M. bovis} BCG. One clone, labelled 531-6, which gave a direct reaction with streptavidin, was isolated and characterised. \textit{EcoRI} digestion of DNA from this clone showed that it contained an insert of approximately 6 kb. Western blot analysis as described above showed a streptavidin-reactive band at approximately 85 kDa (Fig. 1, track C). In this instance, expression of the protein was partly inducible with IPTG: a band at a similar position but with greatly reduced intensity was observed in the absence of IPTG (track D). The position of this band from clone 531.6 corresponded to one of several bands which were visible in an extract of \textit{M. bovis} BCG (track E). The IPTG response suggests that the mycobacterial promoter is either absent or inactive and that the gene is co-transcribed with the beta-galactosidase gene of the vector. However, the apparent absence of a fusion protein suggests that translation is initiated at an independent ribosome binding site. Similar streptavidin-reactive clones have also been detected in a \(\lambda\) gt11 library of \textit{M. leprae} (data not shown).

Thus it seems that these recombinant phages code for mycobacterial proteins which can not only be expressed in \textit{E. coli} but are biotinylated in the foreign host. The reaction with streptavidin interferes seriously with the use of this system for screening mycobacterial gene libraries; due to the strong signal and the frequency of occurrence (approximately one per \(5 \times 10^5\) plaques) all of the positive clones initially selected showed this effect. In order to use this system, it is necessary to eliminate the false positives by a combination of suitable controls and streptavidin-blocking.

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