Rapid testing of antimycobacterial agents and identification of acid-regulated genes in *Mycobacterium* spp. using bioluminescence.

A Thesis submitted for the degree of Doctor
of Philosophy at the University of Leicester

by

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For my parents

Statement of Originality

The accompanying thesis submitted for the degree of Ph.D. entitled "Rapid testing of antimycobacterial agents and identification of acid-regulated genes in *Mycobacterium* spp. using bioluminescence" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester mainly during the period between September 1996 and September 2001.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: Tanvir Shafi Date: 5th June 2002.

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Abstract

Jamila Shafi

Rapid testing of antimycobacterial agents and identification of acid-regulated genes in *Mycobacterium* spp. using bioluminescence.

The WHO has declared tuberculosis 'a global emergency'. New treatments are needed and bioluminescence was investigated in this regard. Bioluminescence has been demonstrated as an ideal tool for a reporter of bacterial viability, detection and gene expression.

The first aim of the project was to develop a method for rapid screening of antimycobacterial drugs. *Mycobacterium smegmatis* and *Mycobacterium aurum* containing *luxCDABE* genes from *Photorhabdus luminescens* generated no light. Bioluminescent strains of *M. bovis* BCG and *M. tuberculosis* were generated although this property was unstable. Studies comparing *lux* genes from *Vibrio harveyi* and *P. luminescens* were done to explain these findings. Bioluminescence could be used to evaluate the activity of antimycobacterial agents against mycobacteria in macrophages using a luminometer and a CCD camera.

A comparison of the alamar blue and bioluminescent assays for rapid estimation of antimycobacterial activity of drugs against recombinant mycobacteria was done. Overall, the bioluminescent assay offered the greatest potential for use in high-throughput screening of novel antimycobacterial agents against mycobacteria.

The second aim of the project was to identify genes from *M. tuberculosis* regulated by acid and/or involved in the acid tolerance response (ATR). Bacterial *lux* was used to assay *in vitro* regulation of expression of two genes, Rv3521c (putative decarboxylase) and *phoPR* (two-component regulator). Induced *lux* expression downstream of P*pd* and P*phoPR* promoters was seen in *M. smegmatis* demonstrating acid-inducibility, but no *lux* expression was seen in *M. bovis* BCG. An ATR was determined in *M. smegmatis* and BCG. A putative knockout mutation of Rv3521c was constructed in *M. smegmatis*. Acid tolerance was not exhibited by this mutant with phosphoric acid as the acidulant but was seen when hydrochloric acid, suggesting different mechanisms are employed to induce tolerance to different acidulants.

Abbreviations

ADC:	Albumin Dextrose Complex
AIDS:	Acquired Immune Deficiency Syndrome
ATR:	Acid Tolerance Response
BCG:	Bacille Calmette Guerin
bp:	Base pair
BSA:	Bovine Serum Albumin
CFU:	Colony Forming Units
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytidine triphosphate
dGTP:	deoxyguanosine triphosphate
dITP:	deoxyinosine triphosphate
dTTP:	deoxythymidine triphosphate
DMSO:	Dimethylsulfoxide
DEPC:	Diethyl pyrocarbonate
DNA:	Deoxyribonucleic acid
DNAse:	Deoxyribonuclease
EDTA:	Ethylenediaminoetetra-acetic acid
FMN:	Flavin mononucleotide (oxidised form)
$FMNH_2$:	Flavin mononucleotide (reduced form)
HIFBS:	Heat Inactivated Foetal Bovine Serum
HIV:	Human Immunodeficiency Virus
HBSS:	Hanks Balanced Salt Solution
HBSS-Hepes:	HBSS plus 2.5 mM Hepes, pH 7.4
IPTG:	Isopropyl-β-D-thiogalactosylpyranoside
Kb:	Kilobase
KDa:	Kilodaltons
MIC:	Minimum Inhibitory Concentration
MOPS:	3-N-morpholino-propanesulphonic acid
$NADP^+$:	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
OADC:	Ovalbumin Dextrose Complex
ORF:	Open reading frame

PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PEG:	Polyethylene glycol
RLU:	Relative Light Units
RNA:	Ribonucleic acid
RNase:	Ribonuclease
SDS:	Sodium Dodecyl Sulphate
SSC:	Saline Sodium Citrate
TB:	Tuberculosis
TE:	Tris-EDTA
tRNA:	Transfer RNA
UV:	Ultraviolet
V:	Volts
W:	Watts
X-Gal:	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside
ZN:	Ziehl Neelsen

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APPENDIX 1.

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CHAPTER 1: INTRODUCTION.

1.1. Background

The following work was sponsored by GlaxoSmithKline. There are serious problems with the existing drug regimens for treatment of mycobacterial diseases. Two approaches to high throughput screening to find new antimycobacterial drugs are: Firstly, to screen libraries of compounds for activity against the whole micro-organism. Secondly, to discover possible drug targets in the micro-organism and to screen compound libraries directly against these targets. Luciferase reporter constructs offer means for assessing of mycobacterial viability in both these areas.

The development and assessment of a high throughput screening method was required in order to assay the thousands of compounds generated by the company. The work described in chapter 3 was aimed to accomplish this through construction of an expression vector based on the *lux* operon. This expression vector would serve as a reporter system for the development of a method for the rapid testing of antimycobacterial drugs.

The section of the work described in chapter 4 was done to identify important targets and identify biologically important environments in mycobacteria. Adaptation to acid may be an important part of the virulence of mycobacteria and hence represents an area of drug targeting. A promoter probe vector based on the *lux* reporter system was constructed for this aim. This vector would be used to assay *in vitro* regulation of expression of two genes identified from *Mycobacterium tuberculosis*, a putative amino acid decarboxylase gene and a two-component regulator gene, which are both thought to be inducible by low pH. The work done in chapter 5 aimed to answer the question as to whether the amino acid decarboxylase gene was involved in the acid tolerance response. This was done through construction of a knockout mutation of the decarboxylase gene in *Mycobacterium smegmatis*.

1.2. A Brief History of Tuberculosis.

The term tuberculosis (TB) was first used in 1839. It was derived from the Latin word *tubercula,* meaning small lump, referring to the small scars seen in tissues of infected individuals. Tuberculosis was also once thought to be a form of tumour or abnormal gland (Bloom and Murray, 1992). In the 1870s and 1880s, many scientists held the view that TB resulted from a combination of poor living facilities, a bad environment and hereditary factors (Bates, 1992; Cohen and Durham, 1995).

In fact, tuberculosis is an ancient disease and has existed in humans since ancient times. TB has been known since the time of Hippocrates (460-370 BC) and was referred to as "phthisis" meaning "wasting away" (Daniel, 1994). Tubercle bacilli have also been found from tuberculous bones and from the remnants of old tuberculosis adhesions in the chest of Egyptian mummies (2400 BC) and tubercles have been found in Stone Age skeletons (Zimmerman, 1979; Joklik *et al.*, 1992; Bloom and Murray, 1992). References to TB can be found in the writings of ancient Babylonia, Egypt, and China (www.state.nj.us/index.html).

The first real breakthrough came in 1882 when Robert Koch announced that tuberculosis was caused by *Mycobacterium tuberculosis*, the tubercle bacilli. Koch from his experiments demonstrated the presence of bacteria and explained how they were transmitted. This finding led to ways of decreasing transmission and in finding effective treatment regimens (Cohen and Durham, 1995). Furthermore, Paul Ehrlich discovered 'acid-fastness' of the tubercle bacilli and introduced a staining technique, that was later modified slightly by Ziehl and Neelsen, and which is still performed in the staining and identification of mycobacteria (Grange, 1988). Later, Theobald Smith (1859-1934) demonstrated that tuberculosis in humans and in cattle was caused by different bacilli and found that immunity in animals could be brought about by injection of an extract of the disease-causing organism (cited from Encarta, 1999).

Lehmann and Neumann introduced the generic name *Mycobacterium* in 1896 meaning fungus-bacterium, derived from the mould-like pellicular growth of the tubercle bacillus on liquid medium, nevertheless mycobacteria do not bare any relation to fungi. *Mycobacterium tuberculosis* and *Mycobacterium leprae* were the only two species grouped within this genus. Bacilli cultured from various animals resulted in the

recognition of four 'tubercle bacilli' namely human, bovine, avian and 'cold-blooded' (Grange, 1988).

It has been suggested that tuberculosis probably occurred in animals well before infection in humans. It was thought the human bacillus of *M. tuberculosis* arose from the bovine form, *Mycobacterium bovis*, as a result of domestication of cattle. Consequently indications of the transmission of tubercle bacilli of *M. bovis* from animal to human in the early 1900s, resulted in compulsory Pasteurisation of milk and thus led to decreases in tuberculosis outbreaks (Youmans, 1979).

TB was epidemic in Europe in the 18th and 19th century resulting in high fatality. The reappearance of TB was the most important cause of death among adults in the 19th century (Dubos and Dubos, 1952). Gaspard Bayle described the damage caused by TB in 900 autopsies. René Laennec described the progression of the disease from the initial tubercle through to its final stages. In 1865, Jean-Antoine Villemin also revealed the infectious nature of TB using rabbits, demonstrating that TB could be transmitted from humans to animals with tuberculosis-infected tissue. Indeed, it was in the early 19th century that research to find a cure for TB began (Grange, 1988; cited from Encarta, 1999).

Initial attempts to develop a vaccine based on attenuated strains against tuberculosis failed. Tests in guinea pigs revealed that the vaccines could progress into tuberculosis and hence preventing its further use (Youmans, 1979). These included the "BOVO" vaccine in 1902 attenuated from the human tubercle bacillus and the "Tauruman" in 1904 that used a combination of human and bovine tubercle bacilli.

However, between 1908 and 1921, Albert Calmette discovered that virulent bovine tubercle bacilli became less virulent when subcultured repeatedly on potato-bile medium. These attenuated bacilli were still able to confer a certain amount of immunity against infection with either bovine or human tubercle bacilli (Calmette, 1923). In 1921, the avirulent strain was used as the Bacillus Calmette-Guérin (BCG) vaccine. A newborn child who was considered particularly at high risk, did not go on to develop TB after oral administration of this vaccine and the vaccine was seen as a great success (Bloom and Fine, 1994). Subsequently, further vaccinations of children against

tuberculosis were performed. Although mass vaccination programs were carried out from the 1930s in Japan, Russia, China, Canada, France and other countries, the United States and Great Britain did not adopt the use of the vaccine until 1940, only when trials indicated BCG's substantial protective action against tuberculosis (Youmans, 1979). However, controlled BCG trials carried out in Georgia found no effective protection against the disease and consequently, the vaccine is not routinely used in the United States (Comstock and Webster, 1969; Bloom and Fine, 1994).

Research was continuing to develop an antibiotic for use against *M. tuberculosis*. Initial attempts in 1940 by Selman Waksman led him to isolate actinomycin, although this antibiotic was effective against tubercle bacilli it proved to be too toxic to use on humans and animals. The beginning of modern antibiotic therapy for TB began in 1944 when Selman Waksman isolated streptomycin from a soil bacterium, *Streptomyces griseus* (Schatz and Waksman, 1944). The administration of this antibiotic to an individual with tuberculosis resulted in maximal inhibition of *M. tuberculosis* with moderately low toxicity (Schatz and Waksman, 1944).

A series of new anti-TB drugs appeared as time went on. Lehmann had discovered para-aminosalicylic acid (PAS) in 1949. Isoniazid (isonicotinic acid hydrazide) was identified in 1952 and was combined with PAS for routine chemotherapy. Pyrazinamide was identified in 1952, cycloserine in 1955, ethambutol in 1961 and rifampicin in 1963. All were introduced as antituberculosis drugs (Doub, 1979).

The successful introduction of drug therapy revealed by the declining rates of disease incidence and mortality over the following 30 years gave hope to the idea that TB could be eradicated. As drug therapy became the primary treatment, mortality rates from TB decreased significantly (Hinshaw, 1979). Deaths from TB in the United States were in excess of 250 per 100,000 in 1882 but by 1900 this figure had dropped to 100 per 100,000. With the introduction of streptomycin the death rate was reduced to 33 per 100,000 in 1946 (Hinshaw, 1979). From 1953 to 1984, the average annual decline in cases was about 5 percent per year. As a result, funding for public health programmes in the United States, including those for the prevention and treatment of TB was drastically reduced in the 1980s (Cohen, 1995).

However, due to over-crowded living conditions, increasing density populations, malnutrition and drug resistance, tuberculosis has re-emerged with greater vengeance and is no longer considered unimportant and intermittent. Furthermore, infected individuals travelling to other countries have resulted in the wider spread of the disease. Thus, tuberculosis has become epidemic and is now a major cause of mortality (Daniel, 1994).

1.3. Microbiology of Mycobacteria.

Mycobacteria are Gram-positive, slightly curved or rod-shaped bacilli that range in size from 0.2 to 0.6 microns in width and 1.0 to 10 microns in length. They are non-motile, non-spore forming, have no capsule, do not produce toxins and are obligate aerobes (Buchanan and Gibbons, 1974). Mycobacteria are found in soil, vegetation, water, warm-blooded and cold-blooded animals. They are comprised of obligate and facultative parasites, and saprophytes with varying nutritional requirements. Saprophytic strains will grow on simple substrates; others require more complex media or additional supplements for growth whilst others will only grow within living cells (Buchanan and Gibbons, 1974).

Mycobacteria have a high cell-wall lipid content and therefore are not readily stainable using conventional bacterial stains, such as the Gram stain. Mycobacteria characteristically stain with the Ziehl-Neelsen (ZN) stain and as such are known as acid-fast. Acid fastness is partially or completely lost at some stage of growth by a variable proportion of the cells of some species; cells of rapid growers may be less than 10% acid fast (Buchanan and Gibbons, 1974). Although acid-fastness is not unique to mycobacteria it does however allow differential staining of contaminated clinical specimens, such as sputum (McMurray, 1998).

In addition, the cell wall of mycobacteria is composed of complex waxes, glycolipids and long-chain fatty acids called mycolic acids. The cell wall consists of several layers; the inner layer of cross-linked peptidoglycan is covalently linked to arabinogalactan, a polysaccharide consisting of arabinose and galactose. The arabinose is esterified at the distal ends to the long-chain mycolic acids. The arrangement of the mycolic acids is perpendicular to the cell surface, forming an inner leaflet of an asymmetric bilayer structure. The outer layer consists of mycosides formed from either peptidoglycolipids

or phenolicglycolipids (Minnikin, 1982; Connell and Nikaido, 1994; Baulard *et al.*, 1999; Grange, 1988). Other cell wall components of importance are trehalose dimycolate, thought to induce growth in serpentine cords on artificial medium and mycobacterial sulfolipids that may well play a role in virulence. Lipoarabinomannan may also contribute to pathogenesis through the ability to stimulate cytokine production in mononuclear cell cultures (McMurray, 1998).

The murein component of the cell wall of mycobacteria confers its use as Freund's complete adjuvant. Further investigations using purified proteins and their corresponding antigens have been demonstrated for use in immune protection alone and in combination with adjuvant formulation to assess protection against *M. tuberculosis* (Horwitz, 1995). No long lasting protection has been demonstrated with this approach although strong type 2 responses have been shown, however for protection, type 1 responses are necessary as these give rise to the cytotoxic phenotype (Bonato, 1998).

The high lipid content of mycobacteria gives rise to increased resistance to chemical injury and especially resistance to physical stress. Mycobacteria are resistant to agents normally used for disinfection such as sodium hydroxide and detergents, they are however sensitive to formaldehyde and glutaraldehyde. They are also resistant to dehydration; they can survive several weeks at 4°C and for several years at –70°C (Shekleton, 1995).

The genus can be divided into two groups, the rapid growers and the slow growers. Some of the rapid growers have a generation time of approximately 2 hours and are classified as forming colonies on solid medium in less than 7 days of incubation at 37°C; these include *Mycobacterium smegmatis*, *Mycobacterium aurum, Mycobacterium parafortuitum* and *Mycobacterium phlei* (Buchanan and Gibbons, 1974). The generation time of slow growers is approximately 18 to 24 hours. They are classified as forming visible colonies after 7 days or longer. *Mycobacterium tuberculosis* and *Mycobacterium bovis* can take up to 3 to 4 weeks to grow on solid medium. Some colonies appear as off-white in colour, waxy, irregular and forming clumps. Carotenoid pigments of some species give rise to yellow or orange colonies, the formation of which may or may not require exposure to light (Buchanan and Gibbons, 1974).

Mycobacterium leprae is an obligate intracellular pathogen that has never been cultured *in vitro*. Most of the organisms isolated in culture from lepromatous tissues appear to be mycobacteria related to *M. avium* complex. Survival and proliferation of *M. leprae* requires a mammalian host. The first demonstration of partial infection and transmission was using the mouse footpad (Rees, 1988). For experimental purposes mycobacterial cells are maintained in the armadillo since there is no extensive animal reservoir of *M. leprae*. The doubling time of *M. leprae* is very slow *in vivo*; it is in the magnitude of 10 to 12 days (Grange, 1988; WHO, 1999) and furthermore, *M. leprae* has an incubation period of approximately five years in man (WHO, 1999).

1.4. *Mycobacterium tuberculosis* Genome.

The recent completion of the genome sequence of the strain *Mycobacterium tuberculosis* H37Rv has made it possible to improve the understanding of the biology of mycobacteria and to begin to develop strategies for finding new therapies.

The final contiguous genome sequence was obtained using several approaches that involved the systematic analysis of sequence from a combination of large insert-clones, including cosmids and bacterial artificial chromosomes (BAC), and also from random small-insert clones from a whole-genome shotgun library (Brosch *et al.*, 1998; Cole *et al.*, 1998). The completed genome sequence was identified as comprising 4,411,529 base pairs, containing approximately 4000 genes, and with a guanine-cytosine content of 65.6% (Cole *et al.*, 1998). Remarkably a large amount of the coding sequence is involved in production of enzymes used in lipogenesis and lipolysis. In total, there are approximately 250 enzymes involved in fatty acid metabolism and more than a 100 enzymes for lipid degradation. This is suggestive of an ability of *M. tuberculosis* to use lipids for growth and maintenance, especially *in vivo* where it may take lipids from the host as its main source of carbon (Cole *et al.*, 1998). The genome also encodes polyketide synthase systems I (example mycocerosic acid synthase) and II (example phenolphthiocerol system) but what is unusual is to find enzymes from the PKS superfamily of higher plants namely chalcone and stilbene synthase. These are associated with synthesis of anthocyanin pigments and flavonoids.

In addition, a whole range of insertion elements have been identified in *M. tuberculosis*, 32 novel IS elements in total, some that have been identified to previous known groups

and some that are unidentified (Cole *et al.*, 1998; Gordon *et al.*, 1999). Insertion elements are mobile genetic elements, which are able to move, influence gene expression and cause chromosomal rearrangement. Many of them have either inserted into intergenic or non-coding regions or clustered, demonstrating the existence of insertional "hot-spots" that prevent certain genes from being inactivated (Cole *et al.*, 1998). This has been demonstrated in *Rhizobium* (Freiberg *et al.*, 1997).

Two new families of glycine-rich proteins with a repetitive structure have also been identified and have been defined as the polymorphic GC-rich sequence (PGRS), which is comprised of multiple copies of the sequence CGGCGGCAA (Cole et *al.*, 1998). The major polymorphic tandem repeat (MPTR) is defined as comprising of the sequence GCCGGTGTTG, or its complement, arranged in tandem copies separated by 5 base pair spacers. The genes for the PGRS and MPTR proteins are involved in encoding glycine, alanine and aspargine rich proteins. They belong to two main groups, designated the PE and the PPE families respectively, and account for approximately 10% of the coding of the genome. There are 99 members of the PE protein family and 68 members of the PPE protein family. It has been suggested that PE and PPE may play a role in antigenic variation, as variations in PGRS using restriction fragment length polymorphism (RFLP) have been demonstrated (Cole *et al.*, 1998; Gordon *et al.*, 1999). Direct support for genetic variation within the PE and the PPE families was obtained by comparative DNA sequence analysis. The gene for the PE-PGRS protein Rv0746 of BCG differed from that in H37Rv by the deletion of 29 codons and the insertion of 46 codons. The mechanisms involved in these differences are known to generate antigenic variation in other bacterial pathogens (Robertson and Meyer, 1992; Cole *et al.*, 1998).

1.5. Mycobacterial Diseases.

The approved names of mycobacteria include 47 mycobacterial species (Skerman *et al.*, 1980; Tsukamura, 1981a; Tsukamura *et al.*, 1981b). Mycobacteria can be classified into two groups, *Mycobacterium* complex and non-tuberculous mycobacteria. Members of the genus that are referred to as "the tuberculosis complex" are *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti* (Wayne and Kubica, 1986) and *Mycobacterium canetti* (Van Soolingen *et al.*, 1997). Non-tuberculous mycobacteria are also referred to as "atypical" and include *Mycobacterium scrofulaceum, Mycobacterium marinum* and *Mycobacterium ulcerans.*

The two major human pathogens of the genus *Mycobacterium* are *M. tuberculosis* and *M. leprae* and they are the cause of tuberculosis and leprosy respectively in man (Grange, 1988). In 1993, the World Health Organisation (WHO) declared TB 'a global emergency' (WHO, 1994). Tuberculosis remains a devastating disease resulting in 3 million deaths per year, with greater than 10 million new cases annually. An estimated two billion people are infected with *M. tuberculosis*, although the majority of these will not develop active TB. Of all the deaths worldwide, 7% are attributed to tuberculosis and the majority of the infected population resides in the developing countries (Raviglione, 1995; Anderson, 1998; Snider *et al.*, 1994).

Leprosy also remains a problem in 24 countries, primarily those in South-East Asia, Africa, and Latin America (Brundtland, 1999). The first recording of leprosy dates back to 600 BC and has been seen in ancient civilisations of China, Egypt and India. There are two types of leprosy, paucibacillary (PB) and multibacillary (MB) leprosy. Leprosy effects mainly the skin and nerves and if untreated can lead to permanent damage causing severe mutilation. However if treatment is provided early into the disease, the disabilities from leprosy are halted (WHO, 1999). Although multidrug therapy is effective, prevents the occurrence of drug resistance and is available free of charge through the World Health Organisation, the incidence of leprosy is 1.4 cases per 10,000 people and at the end of 1998 there were 820,000 new cases detected. This figure is estimated to rise to 2.5 million during the period 2000 to 2005 (WHO, 1999).

Variants of *M. tuberculosis* include *M. bovis, M. africanum* and *M. microti. M. bovis* was isolated from bovines and is most often transmitted through non-pasteurised milk or through contact with infected cattle. *Mycobacterium africanum* was originally isolated from man in equatorial Africa (Grange, 1988). The strain resembles some properties of the bovine type, for example, micro-aerophilic, and some properties of the human type, for example, sensitive to pyrazinamide (Grange 1988). *M. africanum* has been isolated from patients from West Africa, East Africa and from Europe (Viane-Niero *et al.*, 2001). *M. microti* is the vole tubercle bacillus that is rarely encountered and is not naturally virulent in man (Grange, 1988).

The MAC complex includes *Mycobacterium avium* and *Mycobacterium intracellulare.* Also named the MAIS complex that included *Mycobacterium scrofulaceum.* All have

similar properties. They have been isolated from the environment, including soil and water and also from house dust (Reznikov *et al.*, 1971; Sommers, 1979). The infections caused by these organisms are usually pulmonary and occur in individuals with pre-existing lung disease or injury. Disseminated infection is usually limited to immunocompromised patients, particularly HIV-infected individuals (Hopewell, 1994).

Non-tuberculous mycobacteria are found in the environment, from soil and water and are usually harmless saprophytes (Nunn and McAdam, 1988). Approximately 17 species of rapidly growing mycobacteria exist but these rarely, if ever, cause human disease. Exceptions are *Mycobacterium chelonei* and *Mycobacterium fortuitum.* Some of the mycobacteria within this group include *Mycobacterium smegmatis* that is found in the smegma of man, *Mycobacterium aurum* and *Mycobacterium phlei* used in biochemical and genetic studies and *Mycobacterium sphagni* found in sphagnum bogs (Buchanan and Gibbons, 1974; Kazda *et al.*, 1979; Grange, 1988).

1.6. Pathogenesis of Tuberculosis.

Tuberculosis may present itself as primary disease that follows soon after the initial infection of the host or secondary disease that occurs often after endogenous reactivation of the primary infection or due to reinfection with exogenous bacilli. Tuberculosis may present not only in the lungs, but can be found in other organs, including kidney, bone, joints and brain (Grange, 1988). Also, infection of tonsils or the gut can occur, although rarely, through ingestion of contaminated milk and milk products in those parts of the world where bovine tuberculosis has not been eliminated. While *Mycobacterium tuberculosis* can produce infection and disease in many organs of the body, it is commonly the result of dissemination from an initial pulmonary focus (Youmans, 1979). However, contact with tubercle bacilli does not always result in infection and disease (Grange, 1988).

The route of transmission of the tubercle bacilli is predominantly through inhalation of droplet nuclei. These are aerosolised airborne particles containing viable bacilli that are expelled from lungs of individuals with pulmonary tuberculosis. As a result of coughing, sneezing, singing, yelling and talking loudly, the respiratory secretions are disrupted generating the droplets (Shekleton, 1995). The evaporation of the droplet leads to formation of a droplet nucleus, which contains the tubercle bacilli, that is very

stable and settles slowly. A droplet nucleus of 10 μm can carry approximately 3 to 10 tubercle bacilli (Smith and Moss, 1994). Transmission of *M. tuberculosis* is affected by the characteristics of the droplets generated, the environmental conditions to which the droplet is exposed and the conditions under which contact with the new host occur. Only airborne particles of 1 to 5 microns can be inhaled into the alveolus; the larger particles are filtered in the nose and cleared by the mucociliary system (Shekleton, 1995).

In primary tuberculosis the inhaled small droplet nuclei lodge within the alveoli where they are rapidly phagocytosed by alveolar macrophages. The outcome of infection is dependent on factors such as the virulence of the tubercle bacilli and the microbiocidal capability of the alveolar macrophage (Dannenberg and Rook, 1994). The tubercle bacilli multiply within these macrophages, but since the maximum rate of multiplication is slow, the increase in numbers of tubercle bacilli will be slow, thus, the appearance of symptoms due to the infection may require several weeks (Youmans, 1979). An inflammatory cellular reaction will occur once numbers of tubercle bacilli become considerably larger. However, tubercle bacilli continue to replicate, lyse and escape from macrophages and infect neighbouring alveolar macrophages and monocytes that enter the site from the bloodstream, attracted by released bacilli, cellular debris and chemotactic factors of host origin. The monocytes cannot inhibit or destroy the bacilli because they are not yet activated (Dannenberg and Rook, 1994). The bacilli continue to replicate resulting in their dissemination, through the lymphatics and then into the blood stream, spreading through to other organs and tissues of the body including other parts of the lung where oxygen tension is high. (Youmans, 1979; Shekleton, 1995).

After 3 to 8 weeks cell-mediated immunity develops and macrophages become activated, that is, they become capable of inhibiting the multiplication of the bacilli within them. Presentation of mycobacterial antigens by macrophages results in proliferation of T-cells and additional macrophages. The tubercle bacilli surrounded by macrophages and activated T cells become walled off, forming a complex called the granuloma, thus, confining the disease (Dannenberg and Rook, 1994). Calcification of the granuloma forms the Ghon complex. These lesions, containing small numbers of viable tubercle bacilli, become dormant and allow infection to remain latent, although breakdown of the lesion can lead to reactivation of infection (Shekleton, 1995; Rook

and Hernandez-Pando, 1996). The bacilli disseminated into the lymphatics or bloodstream are destroyed by the macrophages (Youmans, 1979).

In 90% of individuals, infection resolves, without symptoms since cell-mediated immunity functions to contain the infection. Of the remaining 10%, 50% will be unable to control mycobacterial replication and disease will continue to develop within the first year as primary TB. In the other 50% disease will occur but later on in life (Diagnostic Standards and Classification of Tuberculosis, 1990). Reactivation of previously acquired tuberculosis (secondary TB) can result from the loss of host resistance from the breakdown of cell-mediated immunity. Various factors may contribute such as HIV infection, malignant disease, diabetes, old age, malnutrition, drug and/or alcohol abuse (Cohen and Durham, 1995). Secondary TB can also come about as a result of reinfection from an exogenous source (Youmans, 1979).

Secondary tuberculosis occurs in a small fraction of exposed individuals leading to liquefaction of necrotic lesions and disease (Dannenberg and Rook, 1994). The extensive necrosis is the result of the destructive nature of the hypersensitivity response that occurs (Youmans, 1979). The high concentration of oxygen and softened caseum promotes the growth of tubercle bacilli thus generating elevated numbers of bacilli. This large antigenic load is toxic to the tissues causing progression of the disease and the destruction of the local tissues, including the walls of the adjacent bronchi. The bronchi become necrotic and rupture, forming a cavity, and subsequently the bacilli and the liquefied caseous material are discharged into the airways and reach other parts of the lung and the outside environment (Dannenberg and Rook, 1994). Activated macrophages are no longer effective in controlling the progression of the large numbers of tubercle bacilli as they cannot function or survive in the liquefied material, probably due to the effects of toxic fatty acids originating from host cells and/or the bacilli (Dannenberg and Rook, 1994; Shekleton, 1995). Although, appropriate antimicrobial therapy can reduce the number of viable bacteria and in time, halt the disease, there is no therapeutic agent to prevent liquefaction (Dannenberg and Rook, 1994).

1.7. Drug Treatment and Resistance in *Mycobacterium tuberculosis*.

In view of the tuberculosis global emergency declared in 1993 by the World Health Organisation (WHO, 1994), new and rapid treatment of tuberculosis is needed. To date,

treatment has mainly been through drug therapy and occurs over a long period of time using a combination of the first line drugs, isoniazid (isonicotinic acid hydrazide), rifampicin, pyrazinamide, ethambutol or streptomycin (Madsen and Cohen, 1995). To treat tuberculosis, three mycobacterial populations have to be targeted (Madsen and Cohen, 1995). To treat rapidly proliferating tubercle bacilli present in the oxygenated extracellular environment of the lung, isoniazid is used, since it is effective in killing the majority of the rapidly growing extracellular mycobacteria. Those present within less oxygenated caseous foci, that are intracellular, usually dormant and in a slightly acidic environment are treated using rifampicin, as it sterilises tissues by killing the persistent mycobacteria; those that are dormant or dividing slowly. Isoniazid and rifampicin are considered as the two most important drugs in the treatment of TB (Ellner *et al.*, 1993). Intracellular mycobacteria within activated macrophages with an acidic environment are treated with pyrazinamide. This drug has no activity however against mycobacteria in non-acidic environments (Madsen and Cohen, 1995; McMurray, 1999; Zhang *et al.*, 1999).

The minimum length of time for treatment of TB is six months. Isoniazid, rifampicin and pyrazinamide are prescribed for two months followed by isoniazid and rifampicin for four months; this is a typical but not a unique regime (WHO, 2000a). The treatment strategy depends on the status of TB and the risk of multi-drug resistant tuberculosis (Madsen and Cohen, 1995). Failure due to non-compliance by the individual or administration of inadequate drug regimens, results in the emergence of strains resistant to the drug(s). Non-compliance is common in those living in nations not capable or reluctant to adopt the WHO-recommended Directly Observed Treatment, Short-course (DOTS). The program prevents the development of multi-drug resistant tuberculosis (MDR-TB) by ensuring the full course of treatment is followed (WHO, 2000a). MDR-TB is defined as due to bacilli resistant to two or more antituberculosis drugs, in particular, isoniazid and rifampicin (Heifets and Good, 1994; Cohen and Durham, 1995). DOTS provide services to enable individuals to adhere to their treatment, to ensure acceptable clinic practices and give additional social services to individuals (WHO, 2000a). Approximately 95% of drug-susceptible cases have been cured of disease using DOTS strategies (WHO, 2000a).

Failure of drug therapy can occur for a number of reasons, from strains that are naturally resistant to antituberculosis drugs, acquiring drug resistant strains through contact with other persons and acquired resistance that is treatment-related (Riley, 1993). It is therefore recommended that the length of therapy is increased and to employ multiple drugs concurrently, as use of single drugs can result in emergence of drug-resistant mutants. With exposure to a single drug irregular drug supply and poor adherence to treatment leads to suppression in the growth of susceptible bacilli but allows the growth of drug resistant organisms. Transmission of these bacilli to other individuals may lead to disease that is drug resistant In addition, single drugs added to an already failing regimen only add to drug resistance and thus should be avoided (Riley, 1993; Davies, 1999).

Tuberculosis rates increased in the West in the late 1980s and the early 1990s (Snider *et al.*, 1994). Additional factors contributed to the resurgence of TB included immigration from areas of high incidence of drug resistance, large increases in the homeless population, drug use, poverty, increased numbers in long-term care facilities, those imprisoned and a decline in public health standards. In fact, in 1995, in the order of 30% of San Francisco's and 25% of London's homeless population had TB. In comparison, the overall occurrence in the United States was 7% and 13% in the United Kingdom (WHO, 2000a).

The recent resurgence of TB has been complicated by the outbreaks of multi-drug resistance tuberculosis (MDR-TB)(Cohn *et al.*, 1997). Outbreaks of MDR-TB in New York City involving more than 230 individuals in 1990 were investigated. Of the isolates, 94% had resistance to both isoniazid and rifampicin and 46% of the isolates were resistant to six or more anti-tuberculosis agents (Driver *et al.*, 1994). In 1991, individuals with positive mycobacterial cultures were assessed and resistance to both isoniazid and rifampicin were identified in 30% of individuals with previous antituberculosis therapy and 7% of individuals without prior treatment (Driver *et al.*, 1994). Strains of *M. tuberculosis* resistant to all first line anti-TB drugs, isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide, have now emerged (Frieden *et al.*, 1993; 1995; Driver *et al.*, 1994; Moore *et al.*, 1997; WHO, 2000b). Treatment of MDR-TB is complex and can take up to two years. One major option is to perform

surgery to remove the majority of the infected tissue. A significantly improved cure rate is possible in patients who have this procedure performed (Madsen and Cohen, 1995).

To complicate matters further, one of the major sources of multi-drug resistance tuberculosis are Human-Immunodeficiency-Virus (HIV)-infected populations. The US Centres for Disease Control and Prevention (CDC) reported seven outbreaks of MDR-TB from 1990 to 1992 and of those, 90% of the individuals were HIV-infected and many of them also demonstrated advanced stages of Acquired Immunodeficiency Disease during diagnosis. In fact, TB is known to progress AIDS in individuals who are HIV-positive (Duncans, 1997). Infection with HIV increases susceptibility to TB due to the weakening of the immune system. About one-third of all AIDS deaths today are caused by TB. It is also believed to multiply the risk of initial infection with *M. tuberculosis* (WHO, 1999). Dormant TB is also reactivated and the probability of developing TB rises from 10% over a lifetime to 8% per year (Selwyn *et al.*, 1989).

The epidemiology of tuberculosis has changed radically in recent years, attributable to HIV-infection. In Africa particularly, as well as other developing countries, coinfection with HIV and *M. tuberculosis* doubles or triples the incidence of tuberculosis at a time when TB control programs are already strained (Smith and Moss, 1994; NAID NEWS, 1996). An increase in tuberculosis in the United States is correlated with infection with HIV and also 21% of drug users with AIDS developed tuberculosis (Smith and Moss, 1994). Additionally, in Latin America, 20% to 30% of individuals with AIDS have tuberculosis. Tuberculosis is seen as one of the commonest opportunistic infections present in individuals with AIDS (Smith and Moss, 1994).

1.8. Failure of the Bacillus Calmette-Guérin (BCG) Vaccine.

The use of a vaccine is potentially an effective way of controlling tuberculosis. The BCG vaccine is a live attenuated vaccine that is currently in use for the vaccination of humans against tuberculosis. In 1992, approximately 100 million newborn infants and children received BCG through the WHO/United Nations Children's Emergency Fund (UNICEF) Expanded Program for Immunisation (WHO, 1992). This vaccine has many benefits. It can be administered to newborn infants or at any other time, it has long-lasting sensitisation that is achieved with a single dose and it is considered safe and

inexpensive. It has also shown some degree of protection against leprosy in Asia, Africa and Latin America (Bloom and Fine, 1994).

The vaccine is extremely effective in the United Kingdom with approximately 80% efficacy but the efficacy of vaccine varies considerably throughout the world (Bloom and Fine, 1994). Vaccine efficacy is expressed as the percentage reduction in risk of disease in vaccinated individuals when compared to comparable non-vaccinated controls (Fine, 1988). Questions have arisen about the efficacy of the vaccine given that in South India the vaccine had 0% efficacy (Bloom and Fine, 1994). The level of effectiveness of the vaccine is also based on the suggestion that BCG is highly effective against primary infection in children and endogenous reactivation of an established infection but is extremely low to almost zero, against reinfection from an exogenous source (Bloom and Fine, 1994). Also, during 1929 in Germany, the safety of the BCG vaccine was put into question when 72 children, from a total of 251, died from tuberculosis. However, it was concluded that is was due to contamination with the virulent tubercle bacilli (Fine, 1988; Youmans, 1979). BCG is considered one of the safest vaccines, provided it is given correctly.

It has been suggested that variations in the efficacy of the vaccine could be due to previous infection with non-pathogenic mycobacteria in the environment leading to some degree of protection against the tubercle bacillus. For that reason, any protection arising from BCG would be masked by the naturally acquired protection of the immune response (Palmer *et al.*, 1966; Palmer *et al.*, 1968). Accordingly, in the United Kingdom the occurrence of environmental mycobacteria is low leading to low levels of exposure and sensitisation and hence BCG effectiveness is higher. In comparison to South India (Tripathy), exposure and sensitisation to environmental mycobacteria is extremely high and consequently the BCG efficacy against tuberculosis is low (Lowrie, 1999).

BCG vaccination is not accepted in the United States since it is considered to be ineffective as demonstrated by the Georgia/Alabama and Puerto Rican studies of the United States Public Health Service (Youmans, 1979). Also, individuals immunized with BCG often have a positive reaction to the tuberculin skin test (PPD) and consequently this reduces the usefulness of the epidemiological and diagnostic value of the test because infection with *M. tuberculosis* cannot be distinguished from a BCG

vaccination. Indeed it is important that individuals should only be vaccinated if they are tuberculin negative (Youmans, 1979; Madsen and Cohen, 1995).

1.9. High-Throughput Screening for Drug Discovery.

With 3 million deaths per year and with the increasing number of cases each year, tuberculosis is still a high-impact disease (Anderson, 1998). Furthermore, the rise in multiple drug resistant *M. tuberculosis* and increased susceptibility of HIV-infected populations to tuberculosis demands the need for improved therapy and hence improved methods for the rapid, high-throughput screening of potential therapeutic compounds (Farmer *et al.*, 1998).

Two significant problems hinder the *in vitro* study of potential compounds against *M. tuberculosis*. Firstly, the slow growth rate means it can take up to 4 weeks to form colonies on solid agar media (Young, 1997). Accordingly, methods requiring the measurement of growth of bacteria are time consuming and therefore at a major disadvantage. Secondly, the potential for laboratory-acquired infection from *M. tuberculosis* requires safe microbiological containment that reduces the efficiency of screening.

Mycobacterium aurum and *Mycobacterium smegmatis* are advantageous over *M. tuberculosis* as model organisms because they are not pathogenic and therefore high containment facilities are not required. In addition, they are rapidly growing mycobacteria (1 to 2 days for *M. smegmatis* and 5 to7 days for *M. aurum*) allowing experiments to be done frequently, thus giving the ability to screen increased numbers of antimycobacterial agents. *Mycobacterium aurum*, in particular, has been shown to predict drug activity against *M. tuberculosis* (Chung *et al.*, 1995).

Several methods have been developed to assess antimycobacterial activity against *Mycobacterium* species. However, these are not always amenable for use in high-throughput screening. For example, standard procedures for assaying activity of drugs using agar and broth dilution methods are used widely but as previously mentioned, they involve long time-scales and therefore are subject to contamination by other microorganisms (Heifets, 1988). Methods based on solid medium have problems associated with adsorption and deterioration of the drug, making MIC (minimum inhibitory concentration) evaluation difficult.

Originally, testing done on solid media included the use of Lowenstein-Jensen egg-based agar (LJ) (Canetti *et al.*, 1963) and Middlebrook 7H11 agar (McClatchy, 1978). These media are still used today to make evaluations, and a number of studies have demonstrated their use. Testing has also been done using liquid medium. MB Redox, a new mycobacterial system recently developed by Biotest (Germany) for the detection of growth of mycobacteria combines a redox indicator with liquid medium to enable growth to be observed microscopically. This system uses a tetrazolium salt that when reduced by the growth of mycobacteria appear as red-to-violet particles. This method was compared to the conventional LJ medium; detection of *M. tuberculosis* on LJ medium required 28.9 days while only 23.6 days was needed for detection using the redox indicator. For nontuberculous mycobacteria, growth was observed in 40.6 days on LJ medium and 32.3 days in MB Redox (Cambau *et al.*, 1999). MB Redox is impractical for use in high-throughput screening because this method relies on microscopic detection rather than, on observable growth. Also MB Redox and growth on LJ medium are impractical because of the increased length of time required for the detection of growth of mycobacteria.

1.9.1. Radiometric Systems.

The radiometric BACTEC 460 TB system is currently widely used for susceptibility testing of mycobacterial clinical isolates (Laszlo *et al.*, 1983; Heifets, 1986; Inderlied and Young, 1988). The antimicrobial drug is added to Middlebrook 7H12 broth, which also contains ^{14}C-palmitic acid and the isolate. This method relies on the release of radioactive carbon dioxide ($^{14}CO_2$) from the labelled precursor by bacterial metabolism, rather than, on observable growth. The air space over the medium is sampled periodically for the release of CO_2; the radioactivity measured is converted into a radiometric growth index (GI), which indicates the number of surviving bacteria. Whilst reasonably fast (results are obtained in approximately 5 days), it requires specialised and costly equipment and necessitates the disposal of radioisotope waste. In addition, it is not suited to high-throughput screening strategies due to the limitations in the number of test bottles that can be sampled at one time. However according to other studies, an average of 18 days is required for drug susceptibility testing of clinical *M. tuberculosis* as compared to 38.5 days using the standard agar proportion method (Roberts *et al.*, 1983).

The BACTEC system has also been used to investigate the susceptibility of *Mycobacterium avium* complex to various antimicrobial agents in order to check accuracy and reproducibility. A 95.7% to 100% reproducibility was observed when 10 strains were tested against eight drugs. The same samples were also plated out to determine the number of colony forming units. In general, both methods were comparable and consequently the BACTEC system has been suggested for use in clinical laboratories and in evaluating new drugs for use against *M. avium* infections (Siddiqi *et al.*, 1993).

A method based on the release of $^{14}CO_2$, but which avoids the need for BACTEC equipment, has also been demonstrated for susceptibility testing. This method relies on the release of $^{14}CO_2$ when ^{14}C-acetate is incorporated into growing *M. tuberculosis* in liquid scintillation vials. The release of $^{14}CO_2$ is measured in a scintillation counter. Nevertheless, this method is again not suitable for high-throughput screening since large numbers of samples cannot be tested, as with the BACTEC 460 TB system (Ashtekar *et al.*, 1987).

A rapid assay method developed for use in high-throughput screening to test for novel molecules that inhibit mycobacterial growth has been described (Chung *et al.*, 1995). *Mycobacterium aurum* was incubated with ^{3}H-uracil in broth and the inhibitory effect of the agent on the viability of the cells was measured by the uptake of radiolabelled uracil after a 6 hour incubation period. Although considered a rapid and reliable method, once more the use of vast quantities of radiolabel in a high-throughput screen presents problems, including the handling and disposal of the radioactivity and therefore this method may not be the appropriate choice for high-throughput screening. However, the use of a lower category organism bypasses the difficulties presented when using a category III organism such as *M. tuberculosis*.

A non-radiometric BACTEC system has been developed. The MB/BacT system relies on a colourimetric CO_2 detection device to indicate mycobacterial growth in a closed system (Rohner *et al.*, 1997). Growth of mycobacteria in the vials leads to production of CO_2 that in turn is sensed by the solid-state sensor at the bottom of each vial that contains a colourimetric indicator that changes from green to yellow. A reflectometer and a detection unit present in each compartment of the instrument allow the measured

values to be transmitted to a computer. Measurement of incubated vials occurs every 10 minutes. Growth of mycobacteria is then measured computationally (Rohner *et al.*, 1997). Its use has been demonstrated in a number of studies to compare its performance against the radiometric BACTEC 460 TB system and against solid medium for the detection of mycobacteria in clinical specimens (Rohner *et al.*, 1997; Benjamin *et al.*, 1998; Brunello *et al.*, 1999). These studies evaluated the MB/BacT system as sensitive, rapid and capable of recovering mycobacteria from clinical samples. However, in a further study to compare MB/BacT with BACTEC 460 TB system and LJ medium was in disagreement these studies (Roggenkamp *et al.*, 1999). The detection of mycobacteria from clinical specimens revealed the MB/BacT system to be less sensitive. In this study, the recovery rate for *M. tuberculosis* isolates were 80.2% compared to 96% in the other studies (Benjamin *et al.*, 1998; Roggenkamp *et al.*, 1999). The sensitivity with MB/BacT was even less with smear-negative specimens with only 78% detection and this method also failed to detect infectious tuberculosis in three patients. Variations in detection times was also noted, with 17.2 days, 15.4 days and 29.8 days being required for MB/BacT, BACTEC 460 system and LJ medium respectively (Roggenkamp *et al.*, 1999).

1.9.2. Flow Cytometry.

Flow cytometry has been reported as a method for susceptibility testing (Bercovier *et al.*, 1987; Mason *et al.*, 1994; Nordon *et al.*, 1995; Ryan *et al.*, 1995; Bownds *et al.*, 1996). This method is based upon a reaction whereby the esterases present in viable *M. tuberculosis* hydrolyse fluorescein diacetate (FDA) to fluorescein, consequently producing fluorescent bacilli that can be detected by flow cytometry. In one study of the technique, after incubation of *M. tuberculosis* H37Ra with four drugs, isoniazid, ethambutol, rifampicin or streptomycin, and addition of FDA, analysis with a FACScan flow cytometer revealed bacilli hydrolysed less FDA resulting in reduced fluorescence than untreated *M. tuberculosis* (Nordon *et al.*, 1995). The MICs demonstrated by this method compared well with agar dilution methods but, although results are available within 24 hours and the method does not rely on the growth of mycobacteria, problems arise due to the clumping of mycobacteria and their relatively small size (Moore *et al.*, 1999; Nordon *et al.*, 1995). Also an issue of safety is a major concern when using this system as live mycobacteria are used and aerosols can be produced during FACScan

analysis because it operates under a pressurised system. Therefore the tests must be carried out in containment facilities (Moore *et al.*, 1999; Nordon *et al.*, 1995).

In order to deal with this concern, viable mycobacteria were treated with 1% paraformaldehyde for 40 minutes to inactivate the cells before commencing flow cytometric analysis (Moore *et al.*, 1999). This step was added when assessing the susceptibility of 17 clinical isolates of *M. tuberculosis* to various antituberculosis drugs and comparing the method to standard agar proportion methods (Moore *et al.*, 1999). General agreement of 98% was demonstrated between the results of the two methods. *Mycobacterium tuberculosis* incubated for 24 or 48 hours in the presence of antituberculosis drugs did not show a decrease in FDA hydrolysis compared to untreated *M. tuberculosis*. Results were obtained within 72 hours of testing when using flow cytometry; this increased length of time was required to obtain optimal results. However, treatment with paraformaldehyde did not modify the results of the susceptibility tests (Moore *et al.*, 1999).

Flow cytometric analysis can be facilitated by encapsulation of mycobacteria in a gel microdrop (GMD). This allows it to behave as a larger organism and provides an environment that prevents cell aggregation (Ryan *et al.*, 1995). This method involves separating the clumps first by vortexing the suspension of cells vigorously, then passing it through a 27.5-gauge needle and eventually filtering the cell suspension through a 5 μm pore size filter to remove any remaining clumps. The gel microdrops are then made by mixing agarose with the prepared mycobacterial cell suspension, followed by emulsification in a CellSys 100 microdrop maker to produce agarose gel microdrops approximately 25 μm in diameter. The GMDs then are washed and analysed in the presence or absence of drugs, which are then stained and fluorescence analysed by flow cytometry. The susceptibility of encapsulated mycobacteria to isoniazid and rifampicin using this method revealed inhibition of growth when compared to cultures grown in their absence (Ryan *et al.*, 1995). In addition, this method also detected isoniazid resistance subpopulations when present in an isoniazid sensitive population. Although this method takes into account the size and clumping of mycobacteria, it requires efficient preparation of the microdrops and thus this method may not be practical for use in high-throughput screening.

1.9.3. Firefly Luciferase.

The cloning of the firefly luciferase gene and its transformation into mycobacteria and other bacteria represents a simple but powerful tool that has allowed it to be used as a reporter system in screening assays to measure the drug susceptibility profiles of clinical isolates and as the basis of high-throughput screens for new antimycobacterials (DeWet *et al.*, 1985; 1986; Jacobs *et al.*, 1993; Cooksey *et al.*, 1993; 1995; Arain *et al.*, 1996a; 1996b; Williams *et al.*, 1999). Firefly luciferase has also been used to screen natural products for antimycobacterial activity against mycobacteria (Shawar *et al.*, 1997). This system is based on production of light from the catalysis of luciferin and ATP by firefly luciferase being from viable bacilli only, thus allowing the effect of antimycobacterial agents to be measured.

Such studies have used luciferase-positive reporter phages in detection and assessment of drug susceptibility in mycobacteria (Jacobs *et al.*, 1993; Sarkis *et al.*, 1995). Mycobacteria were infected with reporter phages expressing the firefly luciferase gene, which was under control of the *hsp*60 heat shock promoter of BCG. Addition of luciferin to the mycobacteria infected with reporter phages resulted in light production within minutes and which increased 1000-fold within 2 hours. However, production of light was abolished with the addition of antituberculosis drugs, isoniazid and rifampicin. These phage were also capable of distinguishing between drug-resistant and drug-sensitive organisms as drug-resistant mutants continued to produce light after the addition of drugs, whilst the parent strain did not (Jacobs *et al.*, 1993; Sarkis *et al.*, 1995). Thus, this is a simple and rapid method and is specific, as only those bacteria infected with luciferase reporter phage will produce light. Furthermore, the intact mycobacteria can take up the substrate, luciferin, without the need for cell lysis and thus simplifying the assay (Jacobs *et al.*, 1993; Hatfull and Jacobs, 1994). Luciferin was added to intact mycobacteria and luciferase activity was found to be measurable (Jacobs *et al.*, 1993). However, the sensitivity of detection is relatively poor, approximately 10^4 to 10^5 cells grown in culture are required to produce a significant signal compared to cells harbouring the luciferase plasmids. This is probably as a result of the effect of the phage on host cell metabolism, poor expression of the luciferase genes or ATP levels (Jacobs *et al.*, 1993). Also, after phage addition, light output peaks for only 2 to 3 hours and then declines because of cell lysis. The mycobacteriophage L5 has a small host range; it can form stable plaques on fast-growing mycobacteria such as *M. smegmatis*

but does not appear to form plaques efficiently on either *M. tuberculosis* or BCG. No temperate phages for these slow-growing mycobacteria have been described (Hatfull and Jacobs, 1994). In addition, the method requires the addition of a costly and freshly prepared substrate, luciferin.

To improve the sensitivity of phage technology, mutants of reporter phages were developed (Carrière *et al.*, 1997). Previously constructed phage either resulted in loss of detectable light and limited the sensitivity of detection to 10^4 mycobacterial cells or failed to efficiently infect and lysogenise *M. tuberculosis*, thus restricting its use (Jacobs *et al.*, 1993; Sarkis *et al.*, 1995). As a result, various luciferase reporter phage (LRP) mutants were constructed but one in particular, phAE88, was shown to be effective in luciferase production even though it replicated only temporarily after infection. Although this mutant was less capable of generating plaques it did, however, produce increased luciferase output that was sustained for a longer period of time, 12 to 24 hours, only decreasing to basal levels at 36 hours. When stationary phase grown BCG or *M. tuberculosis* were infected with LRPs and luciferase was measured after the addition of luciferin, the increased sensitivity allowed as few as 120 BCG cells to be detected. To determine drug susceptibility, mycobacteria were incubated for 24 hours in the presence or absence of drug and then infected with phAE88 for 2 to 4 hours (Carrière *et al.*, 1997). In addition, luciferase activity of drug-resistant BCG cells treated with drugs was also measured (Carrière *et al.*, 1997). The LRPs were able to discriminate between sensitive and resistant strains and the profiles of the drugs against the strains compared well to concentrations used clinically for each antimicrobial agent. Furthermore, the length of time required for the drug susceptibilities studies of *M. tuberculosis* were reduced, such that results were obtained in one day (Carrière *et al.*, 1997).

The phage assay has further been modified for use with the 'Bronx Box' (Riska *et al.*, 1999); this is a custom-made light-tight box, which holds a 96-well microtitre plate and a Polaroid film cassette to allow detection of emitted light. It has been applied to *M. tuberculosis* reference and clinical strains to demonstrate resistance or susceptibility to antituberculosis drugs (Riska *et al.*, 1999). This technique requires exposure of the film to at least 10^5 colony-forming units for a minimum of 3 hours; even then the sensitivity of detection of luciferase on the Polaroid film was low. Generally, this method would

not be practical for high-throughput screening and also it does not produce quantitative results.

Firefly luciferase is also being used to evaluate the activity of antimicrobial agents against *M. tuberculosis* H37Ra and an isolate of *M. avium* A5 with a view to developing a high-throughput screen (Cooksey *et al.*, 1993; 1995). Mycobacteria were transformed with pLUC10, an *E. coli- Mycobacterium* shuttle vector (based on pMV261) carrying the firefly luciferase gene under the control of the *hsp*60 mycobacterial heat shock promoter. Cultures of *M. tuberculosis* were grown to stationary phase, in 7H9 broth with supplements, the cells were washed, diluted and added to a number of antimicrobial agents and then tested for susceptibility. Following incubation of cultures for a period of 2 to 14 days, the cells were lysed and bioluminescence was measured with the addition of luciferin in an ML 1000 Luminometer for 15 cycles. The inhibitory concentrations determined when using the conventional broth microdilution method compared well with the results with luciferase for *M. tuberculosis* and *M. avium*. The expression of luciferase in *M. avium* was 5-fold greater than that in *M. tuberculosis* when equal numbers of intact cells were measured for bioluminescence suggesting there may be differences in the expression of genes between these two organisms.

Studies have also demonstrated the use of recombinant strains of mycobacteria expressing an integrated firefly luciferase gene to determine the activity of antimycobacterial agents in macrophages (Arain *et al.*, 1996a; Arain *et al.*, 1996b). Human THP-1 macrophages were infected with either *M. bovis* BCG or *M. tuberculosis* transformed with the construct pMV361-*lux* (*lux* corresponds to the firefly luciferase gene - normally represented as *luc* - and not bacterial *lux*) (Hickey *et al.*, 1996; Arain *et al.*, 1996b). (The plasmid, pMV361-*lux*, is part of the pMV series but it is an integrative expression vector). After incubation for 4 hours, cells were washed and fresh medium containing isoniazid or rifampicin was added to the macrophages. After 1.5 hours, or until completion of the experiment, the monolayers were lysed and bioluminescence was measured in a Wallac Autolumat LB 953 luminometer after the addition of luciferin. Macrophages infected with BCG, when exposed to isoniazid or rifampicin for a period of 5 or 7-days respectively, demonstrated reduced bioluminescence. However, the results were less distinct when macrophages were exposed to isoniazid or rifampicin for a period of 1.5 hours. Furthermore, although isoniazid and rifampicin were both

observed to be highly effective against intracellular *M. tuberculosis*, rifampicin was shown to elicit a more rapid antimycobacterial response than isoniazid (Arain *et al.*, 1996b). Less time is required to observe the affect of antimycobacterial drugs on the growth of mycobacteria in macrophages compared with measuring growth on solid medium. However, this method still requires the addition of a costly prepared substrate, luciferin.

Animal studies with *M. tuberculosis* undoubtedly are more difficult to accomplish. However, recombinant mycobacterial reporter strains have been developed to evaluate antimycobacterial activity in mice (Hickey *et al.*, 1996). BCG was transformed with the plasmid pMH30 (rBCG:pMH30), an *E. coli-Mycobacterium* shuttle vector carrying the firefly luciferase gene cassette, downstream of a synthetic promoter MOP, consisting of combined elements of the *E. coli tac* and BCG *hsp*70 promoters. Female BALB/c mice were injected intravenously with a sonicated culture of rBCG:pMH30 and after 4 to 24 hours after infection, drugs were administered mainly by oral gavage. Organs were removed, homogenised and luminescence from rBCG-*lux* was measured, without cell lysis, in a luminometer after addition of luciferin. Twenty-four hours after infection with rBCG-*lux*, luminescence was measured and was detected in homogenates of the spleen and liver, but was not detected in lung homogenates, demonstrating that rBCG-*lux* were unable to survive in the lungs 24 hours after intravenous infection. When mice were treated for a period of seven days with antimycobacterial drugs, a reduction in luminescence in the spleen was seen in the drug-treated mice compared to untreated mice. Consequently, this approach allows the assessment of the efficacy of antimycobacterial compounds *in vivo*. Although, this method could be performed in the second stage of drug testing, it is not feasible for high-throughput screening.

1.9.4. Colorimetric Assay.

Colorimetric assays have been used in drug susceptibility testing and in detecting resistant strains of *M. tuberculosis* (Gomez-Flores *et al.*, 1995; Mshana *et al.*, 1998). In one method, the colorimetric assay utilises a yellow dye that changes colour when reduced by living cells. The dye, dimethylthiazolyldiphenyl tetrazolium (MTT) is reduced by mitochondrial dehydrogenase in living cells to produce insoluble formazan (Denizot and Lang, 1986). The possibility of using MTT has been considered in order to detect the viability of *M. tuberculosis* after exposure to rifampicin (Mshana *et al.*,

1998). Viable mycobacteria were found to reduce MTT whilst mycobacteria that were dead were no longer able to reduce MTT. For example, strains of *M. tuberculosis* and BCG sensitive to rifampicin were unable to reduce MTT after exposure to rifampicin for 48 hours. Three resistant strains of *M. tuberculosis* tested continued to reduce MTT after incubation with rifampicin for 48 hours. In addition, the presence of a subpopulation of rifampicin resistant strains was detectable in a population of rifampicin sensitive strains using MTT (Mshana *et al.*, 1998). This method requires lysis and extraction procedures before absorbance can be measured. Also, the MTT assay could not be standardised to detect for isoniazid resistance (Mshana *et al.*, 1998).
A further method that also is based on reduction of a dye as an indicator of bacterial growth is the alamar blue assay (Ortman, 1999). The reduction of alamar blue requires its uptake into the bacterium and this reduction is associated with metabolism occurring within the cells. The alamar blue method is dependent on reducing agents that increase during proliferation of the cell and consequently, as alamar blue accepts electrons, the conversion of oxidised non-fluorescent resazurin (dye) to the reduced fluorescent form, resorufin, by viable bacteria occurs, resulting in a colour change of the dye from blue to pink. The addition of toxic drugs to the bacteria, results in the cessation of metabolic activity and prevents this colour change (Ortman, 1999). Alamar blue reduction can be monitored in a number of ways; visual inspection to observe change in colour, fluorometrically to measure fluorescence or spectrophotometrically to measure absorbance (Ortman, 1999). The alamar blue assay has been used for antimicrobial susceptibility testing of mycobacteria, Gram-negative bacteria, methicillin-resistant *Staphylococcus aureus*, yeasts and enterococci (Novak *et al.*, 1993; Baker *et al.*, 1994; Pfaller and Barry, 1994; Tenover *et al.*, 1995; Yajko *et al.*, 1995; Zabransky *et al.*, 1995; Collins and Franzblau, 1997). Alamar blue has also been used to quantify lymphocyte proliferation (Ahmed *et al.*, 1994).

The susceptibility of 50 different strains of *M. tuberculosis* was tested against a number of antimicrobial agents, including, isoniazid, rifampicin, streptomycin and ethambutol, using the alamar blue assay (Yajko *et al.*, 1995). Cultures of mycobacteria were inoculated with twofold dilutions of each drug and incubated at 35°C for 7 to 14 days. Then alamar blue was added to each sample, which were further incubated at 50°C for 2 hours. MICs obtained by the alamar blue method were in agreement with MIC

susceptibility results obtained by the standard agar proportion method, with a general agreement of 97% between the two methods.

An evaluation of the alamar blue assay in comparison to the widely used BACTEC 460 system has been made for high-throughput screening of compounds (Collins and Franzblau, 1997). MICs of 30 antimicrobial agents against *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. avium* were determined using these two methods. Alamar blue was measured both visually and fluorometrically. Of the 30 antimicrobial agents tested, there was little difference between the alamar blue and BACTEC system for 25 to 27 of the agents using the three mycobacterial strains. Differences between the two methods were seen with 0, 2 and 5 of the 30 antimicrobial agents tested against *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. avium* respectively. Overall, MICs determined either visually or fluorometrically for the alamar blue assay correlated with values determined in the BACTEC 460 system.

A further study tested the performance of the microplate-based Alamar Blue assay (MABA) with 34 clinical *M. tuberculosis* isolates and *M. tuberculosis* H37Rv against isoniazid, rifampicin, streptomycin and ethambutol to determine their MICs (Franzblau *et al.*, 1998). The alamar blue assay was done concurrently with the BACTEC 460 system to compare the results. The alamar blue assay was done using bacterial suspensions that were prepared directly from solid medium rather than liquid grown cultures. The cultures were incubated with each drug, after which alamar blue reagent was added to each sample and incubated at 37°C for 24 hours. Results for all strains were obtained in 8 days; these were measured by visual means only. Some of wells appeared violet after 24 hours of incubation but after an extended incubation period these wells turned pink. Initially results were conflicting with only 88% general agreement, however on repeating some of the tests, 94% agreement was reached. Furthermore, incubation of cultures with alamar blue at 37°C increased the assay time by 2 days (Franzblau *et al.*, 1998). Other studies have demonstrated that alamar blue reagent can change from blue to pink after incubating the cultures for only 2 hours at 50°C (Yajko *et al.*, 1995). Using this method, MIC results were obtained in 7 days for only 58% of the strains of *M. tuberculosis* tested, compared with 8 days to obtain MIC results for 100% of strains of *M. tuberculosis* tested (Franzblau *et al.*, 1998).

The alamar blue reagent has been used in a number of studies as described. It is a useful indicator of cellular growth and because it does not alter the viability of cells and is non-toxic, monitoring can be done continuously (Ortman 1999). It is also non-radioactive and hence is practically easier to perform and also it is cheaper than other methods that are in use, such as the BACTEC 460 TB system.

1.9.5. Bacterial Bioluminescence.

1.9.5.1. The Organisation of the *lux* Operon.

Lux genes from a number of species of marine and terrestrial bacteria have been used to evaluate antimycobacterial activity *in vitro* and as reporters of gene expression (Engebrecht *et al.*, 1985; Andrew and Roberts, 1993; Gordon *et al.*, 1994; O'Gaora *et al.*, 1997; Prest, 1997; Snewin *et al.*, 1999). Bacterial luciferase differs from firefly luciferase in that the luciferase reaction is dependent on the reducing agent $FMNH_2$ and not on the levels of ATP (Hill, 1993). Bioluminescence is based on the production of light from a recombinant bacterium containing the appropriate bacterial *lux* genes. Bioluminescence is produced by the catalysis of a long-chain fatty aldehyde, reduced flavin mononucleotide ($FMNH_2$) and oxygen by bacterial luciferase with emission of blue-green light at 490 nm. A compound that injures a recombinant bacterium containing *lux* genes, to cause a decrease in $FMNH_2$, in turn, results in a decrease in bioluminescence.

Bioluminescence refers to the emission of visible light in living organisms mediated by an enzyme catalyst (Meighen, 1993). Most luminescent bacteria are marine in origin and free-living or associated with other marine organisms in a symbiotic relationship. Luminous bacteria are classified into four main genera: *Vibrio*, *Photobacterium*, *Alteromonas* and *Xenorhabdus* (Meighen, 1988). *Xenorhabdus luminescens* is a non-marine luminous bacterial species. Strains have been found both as symbionts with terrestrial nematodes of the family *Heterorhabditidae* and as isolates from human wounds (Colepicolo *et al.*, 1989). There are three strains of the terrestrial bacterium, *X. luminescens*; these include Hb, Hm and Hw. Two of the strains, Hb and Hm, have been isolated from the gut tracts of nematodes or from the hemocoel of insects (Schmidt *et*

al., 1989). The third strain, Hw, has been isolated from a human wound (Colepicolo *et al.*, 1989; Farmer *et al.*, 1989)

The bioluminescent genes *luxCDABE* are common to all known lux systems and are transcribed in that order (Meighen, 1991, 1993, 1994). The genes coding for the bacterial luciferase subunits (*luxAB*) and the fatty acid reductase polypeptides (*luxCDE*) required for the bioluminescent reaction have been cloned and sequenced from a number of marine bacteria that include, *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium leiognathi* and *Photobacterium phosphoreum* (Belas *et al.*, 1982; Engebrecht and Silverman, 1984; Boylan *et al.*, 1985; Delong *et al.*, 1987; Foran and Brown, 1988; Mancini *et al.*, 1988; Baldwin *et al.*, 1989; Illarionov *et al.*, 1990; Lin *et al.*, 1996). The *lux* genes have also been characterised from the non-marine bacterium, *X. luminescens* (Schmidt *et al.*, 1989; Xi *et al.*, 1991; Meighen and Szittner, 1992).

The bioluminescent reaction is catalysed by bacterial luciferase and involves the oxidation of a long-chain fatty aldehyde, reduced flavin mononucleotide ($FMNH_2$) and oxygen, with the emission of blue-green light at 490 nm (Meighen, 1988). The overall bioluminescence reaction catalysed by luciferase is:

$$RCHO + FMNH_2 + O_2 \longrightarrow RCOOH + FMN + H_2O + Light$$

The oxidation of the aldehyde to the corresponding fatty acid is the primary source of energy for the production of light. The remaining energy is supplied by the oxidation of $FMNH_2$ (Meighen, 1988). The reaction is highly specific for $FMNH_2$. Removal of the phosphate or alteration of the flavin ring decreases the activity significantly (Meighen, 1988).

The *luxA* and *luxB* genes code for the α- and β-subunits of luciferase (Cohn *et al.*, 1985; Johnston *et al.*, 1986). These are two non-identical subunits of 40-44 kilodaltons (KDa) and 55-40 KDa respectively, which form a heterodimeric enzyme with a molecular mass of approximately 80 KDa. The polypeptides have approximately 30% identity in the amino acid sequence (Meighen, 1993). It is suggested that the β-subunit evolved from the α-subunit following gene duplication. Alignment of the α- and β-subunits shows the likely occurrence of a deletion event during the evolution of the β-sunbunit

(O'Kane and Prasher, 1992). Although the subunits are homologous, they have different properties. Studies of subunit recombination and protein sequence alignment, have demonstrated a function for the α-subunit. The active site is principally located on the α-subunit, it is a single catalytic flavin-binding site (Meighen *et al.*, 1971; Murata and Lee, 1987). Further studies have suggested that there may in fact be two distinct flavin-binding sites (Vervoort *et al.*, 1986). The β-subunit is essential for the emission of light. Evidence suggests that the subunit can affect the interaction of the enzyme with the reduced flavin (Meighen and Bartlet, 1980). The amino acid sequence of the β-subunit also greatly affects the thermostability of *V. harveyi* luciferase (Escher *et al.*, 1991).

The *luxC*, *D* and *E* genes code for the three polypeptides, reductase, transferase and synthetase respectively, to form the fatty acid reductase complex. This complex is responsible for the synthesis of the substrate aldehyde from the reduction of fatty acids; the aldehyde is required for the bioluminescent reaction (Meighen, 1988). The transferase subunit catalyses the transfer of activated fatty acyl groups (such as, acyl-ACP, acyl-CoA) to water and to other acceptors, with the enzyme being acylated during the course of the reaction. The substrate cleaved is tetradecanoyl-acyl carrier protein (ACP). The synthetase activates the fatty acid, resulting in the formation of a fatty acyl-AMP intermediate that is bound tightly to the enzyme. In the presence of reductase, the acyl group is transferred initially to the synthetase and then to the reductase before being reduced by NADPH to aldehyde (Meighen, 1991, 1993). Long-chain aldehydes are essential for the bioluminescent reaction. The aldehyde of preference, that also appears to be the natural substrate for the bioluminescent reaction in both *V. harveyi* and *P. phosphoreum* luciferases, is tetradecanal. Nonanal and decanal and some unsaturated aldehydes have also been shown to produce high levels of light (Meighen, 1988).

Although, the *luxCDABE* operon is common to all *lux* systems, some differences do exist between the bacteria because of the presence of additional *lux* genes. The bacterial *lux* genes and their organisation in the genome of different bioluminescent species are shown in Figure 1.1. In *V. fischeri*, upstream of *luxC*, there are two regulatory genes, *lux*I and *lux*R. Transcription of *lux*I is required for the production of a small diffusible signal molecule N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which activates LuxR, resulting in the transcription of the *lux* operon (Swift *et al.*, 1994; Dunlap, 1999). The regulatory element is a 28 KDa protein encoded by *lux*R but it is transcribed in the

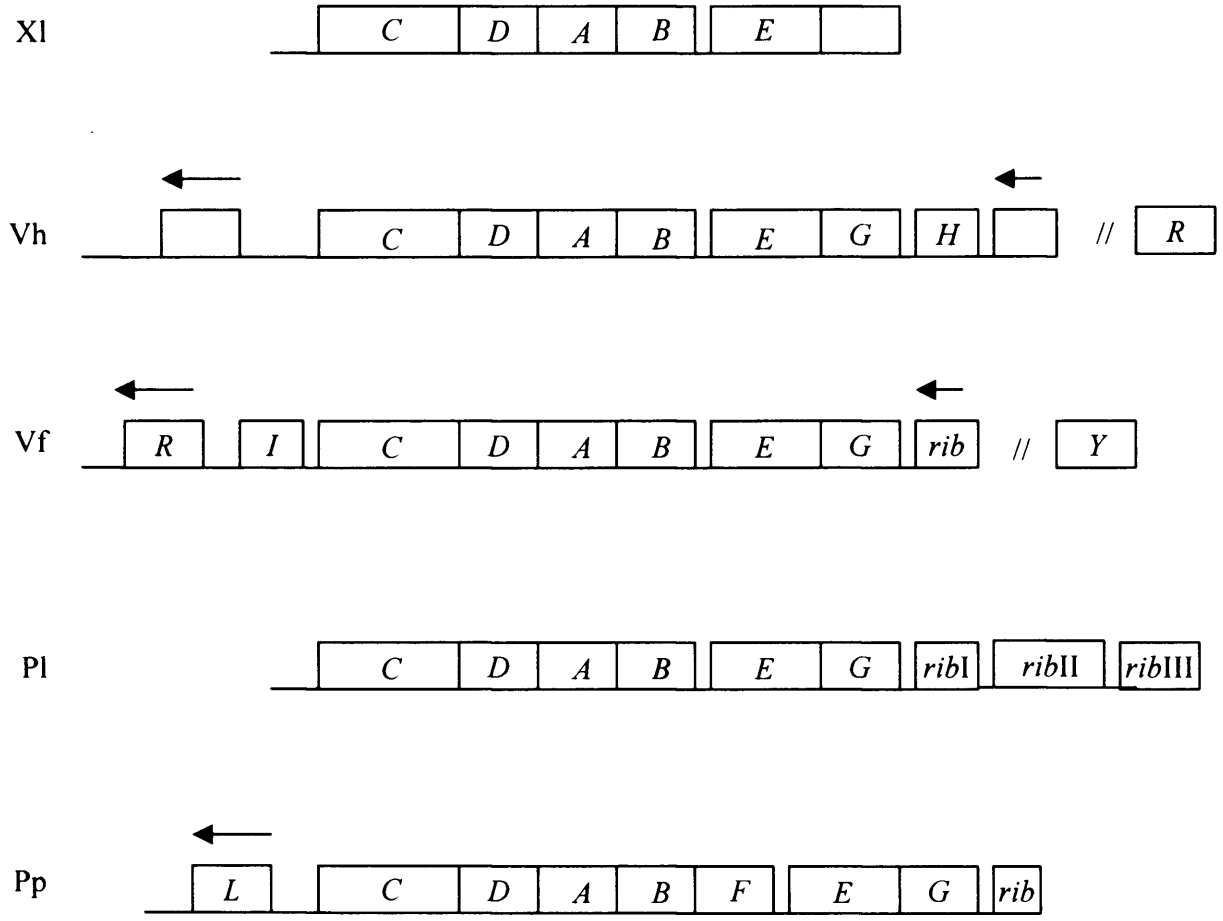

Figure 1.1. The organisation of *lux* genes for *Xenorhabdus luminescens* (Xl), *Vibrio harveyi* (Vh), *Vibrio fischeri* (Vf), *Photobacterium leiognathi* (Pl) and *Photobacterium phosphoreum* (Pp).

The luciferase genes (*luxAB*), fatty acid reductase genes (*luxCDE*), regulatory *lux* genes (*luxR* and *luxI*), flavoprotein (*luxF*), electron transport (*luxG*), riboflavin synthesis genes (*rib* and *luxH*), unlinked *lux* genes Vf *luxY* and Vh *luxR* and empty boxes are open reading frames not yet identified. After Meighen, 1993.

opposite direction to the other *lux* genes (Choi and Greenberg, 1992). *Vibrio fischeri* does not emit light constitutively but exhibits a cell density-dependent process; bioluminescence is only observed once cells reach the mid-logarithmic phase of growth. A sufficient level of homoserine lactone (HSL) in the medium is required before auto-induction is triggered (Stewart and Williams, 1992). A positive feedback loop occurs in which basal transcription of *lux*I leads to the accumulation of a low level of HSL. A LuxR-HSL complex stimulates transcription of the rightward operon. This leads to the additional production of LuxI since *luxI* is the first gene in this operon; the increased HSL further activates LuxR. Because HSL is freely diffusible, the induction of one cell leads directly to the induction of others, creating a positive feedback circuit that can generate a large and rapid response to a small initial stimulus providing a co-ordinated response from a population of cells (Stewart and Williams, 1992; Meighen, 1994; Sitnikov *et al.*, 1995). HSL has also been found to be involved in cell density-dependent carbapenem antibiotic production of *Erwinia carotovora* and elastase production in *Pseudomonas aeruginosa* (Williams *et al.*, 1992; Jones *et al.*, 1993).

The genetic control involved in *V. fischeri* and *V. harveyi* is different although there is a high level of conservation between the structural genes for bioluminescence (Meighen, 1991). At high cell densities, *V. harveyi* is autoinducible by the acyl HSL N-(3-hydroxybutanoyl)-L-homoserine lactone (HBHL) (Cao and Meighen, 1989; Meighen, 1991). LuxO, a repressor of the *luxCDABEGH* operon has been found. Phosphorylation of this protein derepresses the operon, thus allowing transcriptional activation by LuxR. The *luxR* of *V. harveyi* does not respond to the autoinducer HBHL and does not impose density dependent control on the *lux* operon (Showalter *et al.*, 1990). The accumulation of acyl HSL controls LuxO phosphorylation and consequently derepression (Bassler *et al.*, 1993; Bassler and Silverman, 1993).

In *P. phosphoreum* and *P. leiognathi*, *luxE* (coding for fatty acid synthetase) is separated from *luxB* by another gene that has been referred to as *luxF* (Mancini *et al.*, 1988) (also known as *luxN* (Baldwin *et al.*, 1989) and *luxG* (Illarinov *et al.*, 1990)). This gene encodes a flavoprotein and is known as non-fluorescent flavoprotein (NFP) (O'Kane and Prasher, 1992). The flavin prosthetic group is composed of flavin mononucleotide covalently linked to tetradecanoic acid. The LuxF protein (23 KDa) is homologous with the β-subunit of luciferase and has been suggested to evolve as a

result of gene duplication in the *lux* operon (Soly *et al.*, 1988; Baldwin *et al.*, 1989). Approximately 100 amino acids have been deleted to form *luxF*. No functional role of NFP has been demonstrated (O'Kane and Prasher, 1992). However the function may be related to the physiological and/or environmental niche of certain *Photobacterium* species, since it has not been found in *Vibrio* or *Xenorhabdus* strains and is apparently not necessary for the expression of bioluminescence (Meighen, 1991, 1993).

Other *lux* genes have also been identified for example, *luxG* and *luxH* that code for 25 KDa polypeptides of unknown function (Swartzman *et al.*, 1990). In some marine species, the *luxG* gene is closely linked to the *luxE* gene with its ribosomal binding site located in the *luxE* coding region. Transposon insertions in *luxG* of *V. fischeri* and *V. harveyi* do not disrupt the regulation or expression of bioluminescence (Engebrecht *et al.*, 1983; Martin *et al.*, 1989). However, the gene is related to that of flavin reductase and other enzymes involved in electron transport, implicating *luxG* in the production of $FMNH_2$ for the bioluminescent reaction (Meighen, 1993). *luxG* may also code for a function associated with the marine environment because it is not found in the terrestrial bacteria, *X. luminescens* (Meighen, 1991). The *luxH* gene has only been found in *V. harveyi*, where it is located immediately after *luxG* (Swartzman *et al.*, 1990). The gene has significant sequence similarity to *ribB* in *Escherichia coli*, which codes for an enzyme involved in the riboflavin biosynthetic pathway (dihydroxy-4-butanone phosphate (DHBP) synthetase), thus implicating the *luxH* gene in riboflavin synthesis. The *rib-EBHA* genes are also found closely linked in *P. phosphoreum* and *P. leiognathi* downstream of *luxG*. The protein products have riboflavin synthetase, DHBP synthetase, lumazine synthetase and GTP cyclohydrolase II activities, respectively (Meighen, 1994).

Two other genes, *luxL* and *luxY* in *P. phosphoreum* and *V. fischeri* respectively, code for proteins with 30% sequence identity to an enzyme catalysing the conversion of lumazine to riboflavin (Meighen, 1993). The LuxL protein binds lumazine or riboflavin and can affect the wavelength, intensity and decay of light produced by bacterial luciferase resulting in a shift of the light to wavelengths shorter than 490 nm (Meighen, 1991, 1993). In *P. phosphoreum* the *luxL* gene is located upstream of the *luxCDABE* operon and is transcribed in the opposite direction. In some *V. fischeri* strains the *luxY* gene codes for a yellow fluorescence protein (YFP), which is not linked to the *lux*

operon. The YFP causes emission of a yellow rather than a blue-green light (Eckstein *et al.*, 1990).

1.9.5.2. Uses of Bacterial Luciferase.

Bacterial luciferase has been employed to construct a bioluminescent *Mycobacterium smegmatis*, which was used to test susceptibility to various antibiotics and biocides (Andrew and Roberts, 1993). To generate pPA3, the *luxAB* genes from *Vibrio harveyi* were cloned into the *E. coli-Mycobacterium* shuttle vector pMV261 (Stover *et al.*, 1991) where it was under control of the *hsp*60 gene promoter. pPA3 was subsequently transformed into *M. smegmatis* to give a bioluminescent phenotype. Bioluminescent *M. smegmatis* was incubated with gentamicin, chloramphenicol or streptomycin for 24 hours and for 10 minutes with biocides; bioluminescence was measured in a Lab-Line ATP photometer after the addition of decyl aldehyde, the substrate required for the luciferase reaction. Gentamicin and streptomycin revealed major reductions in bioluminescence with complete loss of viability over a period of 24 hours. An 87% reduction in cell viability and 93% reduction in bioluminescence were observed with chloramphenicol. Again, with increasing concentrations of biocides, reductions in viable counts and bioluminescence were demonstrated. Using this system mycobacterial culture is not required and because light production can be measured in real time, the results are obtained earlier than other methods that require bacterial culture before results can be obtained. This study also suggested its use in determining antimycobacterial susceptibility to agents.

The mycobacterial bacterial luciferase reporter constructs have also been assessed in the mouse and in macrophages (Snewin *et al.*, 1999). Recombinant bioluminescent *M. tuberculosis* was constructed by placing *luxAB* genes from *V. harveyi* under the control of the BCG *hsp*60 gene promoter in an *E. coli-Mycobacterium* shuttle vector, pOLYG. The resulting clone, pSMT1, confers hygromycin resistance and thus differs from pPA3, which confers kanamycin resistance. The construct was transformed into *M. tuberculosis* which was shown to become bioluminescent on addition of decanal. The murine macrophages, J774A.1 and female C57BL/6 mice were infected with the recombinant mycobacteria. Results suggested the viability of mycobacteria within macrophages and in the animal model could be monitored by the expression of the bacterial *lux* genes, thus this method could be applied in further study of susceptibility

of mycobacteria to drugs in these models. Also, this would reduce the time required for viable counting, as results would be measured immediately. In fact, these constructs do not have to be integrated because the constructs were stable even in the absence of antibiotic selection; more than 90% of the colonies isolated in this study from the infected tissues remained antibiotic resistant (Snewin *et al.*, 1999).

The entire *lux* operon from *V. fischeri* has been used to generate a mini-Mu*lux* transposon (Engebrecht *et al.*, 1985). Mutations are induced by insertional inactivation by the transposon and the target gene expression can be monitored as a function of bioluminescence. However, there is a limited temperature range for the use of *lux* genes derived from *V. fischeri*. The enzyme is stable in *E. coli* at 30°C but loses activity rapidly at 37°C, which can cause restrictions in some applications, such as, the study of gene expression in animal pathogens (Meighen, 1991). However, the *V. harveyi luxCDABE* system and the *X. luminescens luxCDABE* system give very high light intensity at 37°C in *E. coli* in similar pT7 plasmid constructions. The enzymes encoded by *X. luminescens* are also functional at temperatures as high as 45°C (Szittner and Meighen, 1990).

To allow measurement of bacteria and to eliminate the necessity for the addition of aldehyde, the complete *lux* operon, *luxCDABE*, is employed (Marincs and White, 1994; Winson *et al.*, 1998). Addition of aldehyde to bacteria containing the recombinant *luxAB* genes is not required for light emission if the *luxCDE* genes responsible for aldehyde synthesis are also transferred (Meighen, 1991). Since the aldehyde precursors are probably intermediates or end products in fatty acid biosynthesis (for example, tetradecanoyl-ACP), they should be available in most if not all bacteria (Meighen, 1991). The *luxCDABE* genes from *P. luminescens* (Hb strain) were used to construct a range of plasmids and mini-Tn5 vectors for use in Gram-negative bacteria (Winson *et al.*, 1998). This study demonstrated the ability to screen for mutants of *Chromobacterium violaceum* and *Aeromonas hydrophila* that exhibited growth phase variation in gene expression measured using automated luminometry and photometry in a high-throughput microplate format. Shotgun cloning of *P. luminescens* genomic DNA into plasmid vectors yielded highly bioluminescent *E. coli* in the absence of exogenous aldehyde. Subsequently, a *luxCDABE* cassette was constructed in order to generate plasmids and mini-Tn5 constructs containing the *lux* cassette. Using these constructs,

mutants were identified in *C. violaceum* and *A. hydrophila*. In addition, bioluminescence was constitutively expressed in some of the recombinant organisms containing *luxCDABE* genes, thus, demonstrating *luxCDABE* from *P. luminescens* can be used as a reporter of gene expression (Winson *et al.*, 1998).

The entire *lux* operon from *P. luminescens* has also been used to develop a method for detecting bacterial pathogens in a living host and to evaluate disease processes for strains of *Salmonella typhimurium* that differ in their virulence for mice (Contag *et al.*, 1995). Three strains of *Salmonella* were transformed with a plasmid, pCGSL1, conferring constitutive expression of bacterial luciferase. Mice inoculated with bioluminescent *S. typhimurium* were monitored externally by measuring transmitted photons detected by a CCD detector. Daily injections with carbenicillin were given to select for maintenance of the *lux* encoding plasmid. Bioluminescence was measured over several days and bioluminescent *Salmonella* were observed in specific tissues of the mouse where the bacteria had localised. No bioluminescence was detected from *Salmonella* in the gastrointestinal tract. This is due to the anaerobic environment; oxygen is an essential substrate for the luciferase reaction. Injection of oxygen into the anaerobic area resulted in detectable photons near the injection site demonstrating the presence of bacteria. In addition, a mouse infected with bioluminescent *Salmonella* was treated with the antibiotic ciprofloxacin, known to be effective against systemic *Salmonella* infection. Bioluminescence was measured over 5.5 hours and was reduced to undetectable levels during this period in the treated mouse compared to the untreated mouse, whereby bioluminescence increased 7.5 fold (Contag *et al.*, 1995).

Bioluminescence has been demonstrated as an ideal tool for a reporter of bacterial viability, detection and gene expression because of its non-destructive and non-invasive nature, high sensitivity and real time analysis. The potential for background contamination is low since only organisms containing the *lux* genes or those that are naturally bioluminescent, of which there are few, will produce light. Thus, permitting the extensive use of *lux* genes as reporters of gene expression and in the evaluation of antimycobacterial activity. This system could also be used to generate a construct containing the complete *lux* genes, *luxCDABE*, under control of a suitable promoter and introduced into mycobacteria for use as a reporter gene for antimycobacterial testing. This would allow continuous measurement of bacteria without the addition of aldehyde.

No study has yet reported the use of the complete *luxCDABE* operon from *P. luminescens* in mycobacteria.

1.10. Acid Stress in Bacterial Cells.

Bacteria frequently encounter a number of stress conditions during their existence in the environment and *in vivo*. These include oxidative, temperature, osmolarity, pH stress and nutrient limitation (Foster and Hall, 1990; Ferenci, 1999; Phadtare *et al.*, 1999; Storz and Imlay, 1999; Yura and Nakahigashi, 1999). The survival of the bacteria within these conditions depends on their ability to withstand and survive these stresses. The ability to sense and respond to these stresses will give added protection and may contribute to its virulence.

Maintenance of cytoplasmic pH within a narrow range is necessary to all living cells although this range in internal pH is diverse in bacteria; an internal pH in the range 6.5 to 7.0 is found in acidophiles, pH of 7.5 to 8.0 in neutrophiles and a pH of 8.4 to 9.0 in alkalophiles. The regulation of internal pH for these groups of bacteria requires control over the permeability of the cell membrane to protons (Booth, 1985). Passive movement of protons across the cytoplasmic membrane and the production of acids and bases in the cytoplasm are seen as the main factors causing disturbance of internal pH. Disturbances of internal pH have also been attributed to metabolic processes occurring within the cells. Those bacteria that cannot rely on proton movement alone for regulation of internal pH have evolved additional mechanisms. These mechanisms include cytoplasmic buffering of the cell, production of acids and bases and active transport that involves the antiport system, proton-translocating ATPase (H^+-ATPases) and the electron transport chain (Booth, 1985; Padan and Schuldiner, 1986).

Further extreme acid stress in bacteria would normally lead to acidification of the cell cytoplasm resulting in cell damage and eventually death. However an additional system has evolved in response to an encounter for a short period of time to mild acidic pH. This adaptation to mild acidic pH results in the organism's ability to survive further exposure to lethal acidic pH. This phenomenon is known as the acid tolerance response (ATR). An acid tolerance response has been demonstrated in an increasing number of bacteria; *Aeromonas hydrophila* (Karem *et al.*, 1994), *Enterococcus hirae*, *Streptococcus mutans* (Belli and Marquis, 1991), *Escherichia coli* (Goodson and Rowbury, 1989), *Helicobacter pylori* (Mooney *et al.*, 1990), *Lactococcus lactis* (O'Sullivan and Condon, 1997), *Listeria monocytogenes* (Kroll and Patchett, 1992), root nodule bacteria (Glenn and Dilworth, 1994), *Mycobacterium smegmatis* (O'Brien

et al., 1996), propionibacteria (Jan *et al.*, 2000), *Salmonella typhimurium* (Foster and Hall, 1990) and *Vibrio parahaemolyticus* (Wong *et al.*, 1998). The acid tolerance response in bacteria is not only important for survival in the environment but may also be involved in the virulence of bacteria. In addition, the acid tolerance response has been demonstrated to provide cross-protection against other stresses in the environment suggesting an overlap of the mechanisms maybe involved.

1.11. The Acid Tolerance Response of *Salmonella typhimurium.*

The existence of a global regulatory system for the regulation of internal pH has been identified in *Salmonella typhimurium*. *S. typhimurium* was chosen as a model to study acid survival because of its well-defined genetics and its ability to endure a variety of acidic conditions and therefore most is known about this system in *S. typhimurium* (Foster, 1995). *Salmonella typhimurium* encounters acid conditions during infection, in the host digestive system and in macrophage phagolysosomes. Acid conditions are also encountered in the environment of contaminated ponds and soil (Foster, 1995). They prefer to grow in pH environments above pH 5.5 but can survive in pH as low as 4.0. The inducible acid tolerance response of *S. typhimurium* allows the survival of this organism at further extreme acid conditions. Two types of acid tolerance responses are demonstrated in *S. typhimurium*; the log-phase acid ATR and the stationary-phase ATR (Foster, 1995).

The log-phase acid tolerance response in *Salmonella* is a two-stage overlapping process (Foster, 1995). In general, when actively growing cells are shifted from an external pH 7.7 to conditions below an external pH 4.0, the cells rapidly die. However, the adaptation of the organisms to a relatively mild acidic external pH of 5.8 for one doubling is known as pre-acid shock and is the first stage in the acid tolerance response (Foster and Hall, 1990; Foster, 1995). This involves the synthesis of emergency pH homeostasis systems that alkalinise the cytoplasm during periods of mild acid stress. The second stage, known as the post-acid shock, occurs once external pH falls to or below 4.5 and increases the organism's acid tolerance to further challenge at an extreme acid external pH of 3.3. The post-acid shock depends on the initial adaptation of the pre-acid shock (Foster and Hall, 1990; Foster, 1995; Bearso, 1997). Survival at post-acid shock requires the synthesis of approximately 50 acid shock proteins (ASP) that are induced during the two acid shock stages. These proteins are believed to prevent and/or

repair macromolecular damage caused during post-acid shock (Foster, 1995; Bearso, 1997). Although both stages are required for maximum acid tolerance, a single acid shock at an external pH of 4.3 for 15 to 20 minutes has been demonstrated to give rise to acid tolerance when challenged with an other-wise lethal pH of 3.3 termed acid shock (Foster, 1993). The stages involved in acid adaptation are summarised in Figure 1.2.

Induction of pH homeostasis alone will not afford maximum protection to the organism without the production of ASPs. The majority of the ASPs are produced during the post-acid shock stage. However, reports have demonstrated that the induction of emergency pH homeostasis at pH 5.8 is necessary to permit the production of the ASPs during extreme pH (Foster, 1995; Bearso, 1997). The addition of the protein synthesis inhibitor chloramphenicol, to cells after adaptation to pH 5.8 but prior to challenge at lethal pH 3.3, prevented acid tolerance. Thus, adaptation at pre-acid shock alone is not efficient in demonstrating an ATR, production of ASPs are required in post-acid shock. The importance of the ASPs was further demonstrated. The addition of chloramphenicol to cells that had undergone acid shock at pH 4.3 gave rise to acid tolerance when cells were further challenged at a lethal pH of 3.3 as a result of production of ASPs (Foster, 1995). However, ASPs were only transiently produced at acid shock, after 30 to 40 minutes at pH 4.3 protein synthesis ceased and the organism was no longer able to survive at the lethal pH 3.3 (Foster, 1993).

Given that protein synthesis is reduced when the internal pH is below 6.0, the pH has to be maintained above pH 6.0 for maximum protein synthesis (Foster, 1993). Of the 43 inducible ASPs identified by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis, lowering internal pH induced 22 ASPs, 13 ASPs were induced at an external pH at or below 4.5, whilst the inducing signal for 8 of the APSs was not identified. This suggests that two or more different sets of ASP genes and regulatory systems are present which are based upon a response to internal or external pH (Foster, 1993).

Growth of *S. typhimurium* into the stationary phase is associated with increased resistance to low pH. However, two systems are involved in the stationary phase, one is

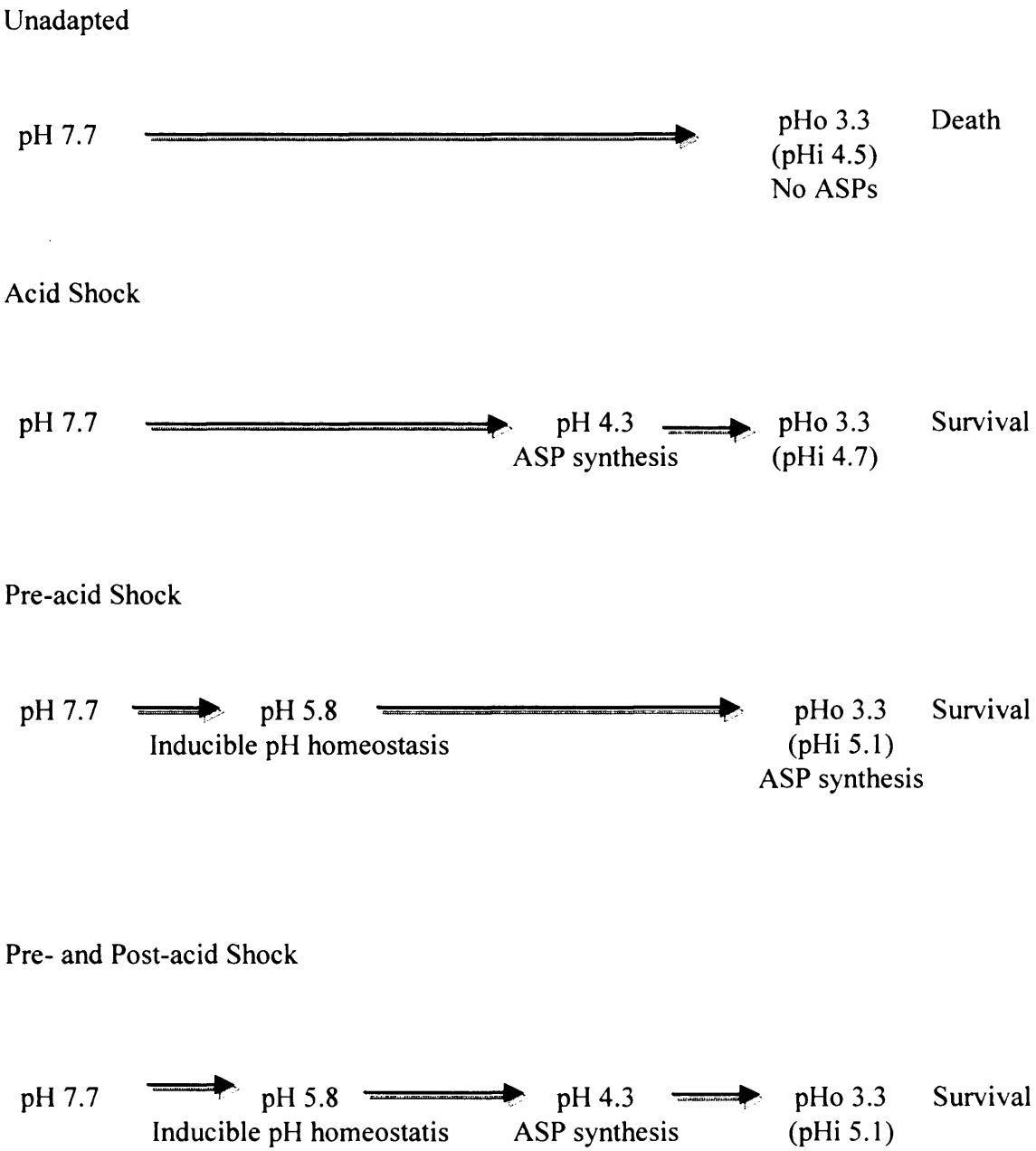

Figure 1.2. Acid Tolerance Response (ATR) of *Salmonella typhimurium.*

(After Foster, 1993)

pHo Extracellular pH

pHi Intracellular pH

ASP Acid Shock Protein

a general stress resistance system that is not dependent on low pH but involves the alternative sigma factor (RpoS) (Lee *et al.*, 1994). The second system is a low pH dependent inducible ATR system that is independent of RpoS; cells grown at pH 8.0 in minimal medium followed by acid shock at pH 4.3 for a period of 2 hours with further acid challenge at pH 3.0 had increased survival compared to cells not adapted to acid shock. Greater protection and survival was observed in the stationary phase ATR compared to log phase ATR and also protection occurred for a longer period of time (Lee *et al.*, 1994). Stationary phase ATR involved the induction of only 15 acid shock proteins as identified by two-dimensional PAGE analysis compared to approximately 43 in log phase ATR (Lee *et al.*, 1994; Foster, 1993). Gene mutations affecting log phase ATR had little or no affect in stationary phase ATR implicating major distinctions between these two systems (Lee *et al.*, 1994; Foster, 1995).

1.12. The Acid Tolerance Response of Other Bacteria.

The acid tolerance response is not only observed in *S. typhimurium* but has been demonstrated in a number of bacteria. This section will present an overview of the acid tolerance response found in various bacteria.

1.12.1. *Escherichia coli.*

Log phase and stationary phase acid tolerance mechanisms exist in *E. coli* but variations between *E. coli* and *S. typhimurium* do occur in response to acid stress. The log phase acid tolerance response in *E. coli* is also termed habituation (Goodson and Rowbury, 1989). Cells habituated to acid for 5 minutes at pH 5.0 in nutrient broth survived better than cells grown at pH 7.0, when challenged at a lethal pH of 3.0 or 3.5. *Escherichia coli* grown in minimal medium required 30-60 minutes at pH 5.0 for acid habituation (Raja *et al.*, 1991). However, non-habituated *E. coli* grown in minimal medium was found to have increased acid resistance compared to organisms grown in nutrient broth indicating inherent resistance must exist. Acid habituation was dependent on protein and RNA synthesis. The addition of chloramphenicol (protein synthesis inhibitor) or rifampicin (RNA synthesis inhibitor) prevented organisms gaining acid-resistance (Raja *et al.*, 1991). Nevertheless, organisms briefly pre-exposed (2.5 minutes) at pH 5.0 were not affected by chloramphenicol suggesting that they were fully habituated. Unlike *Salmonella*, protein synthesis was necessary at pH 5.8 and below pH 4.5 for full acid tolerance (Foster, 1993; Rowbury and Goodson, 1993). In addition, the ATR of

Salmonella was found to be dependent on the products of the *atp* operon (encoding H^+-translocating ATPase) and *fur* gene (encoding ferric uptake regulator) but were not found to be involved in the habituation of *E. coli* (Rowbury and Goodson, 1993).

Acid habituation was reported to be strongly influenced by phosphate (Rowbury *et al.*, 1992; Rowbury and Goodson, 1993). Induction of acid habituation depends on hydrogen ions crossing the outer membrane via the PhoE porin and allowing acidification of the periplasm and hence activation of a sensor. High levels of phosphate could prevent habituation by competing with the hydrogen ions for the PhoE pore and blocking hydrogen ion influx and thus interfering with signal transduction (Rowbury *et al.*, 1992; Rowbury and Goodson, 1993). Addition of phosphate to *E. coli* grown at pH 7.0 prevented killing of the organism when challenged to pH 3.5. *Escherichia coli* strains lacking PhoE pores were very acid resistant and also lost habituation at pH 5.0, confirming the involvement of the PhoE pore in proton transport (Rowbury and Goodson, 1993).

The secretion of extracellar components into the medium of logarithmically grown acid tolerant *E. coli* at pH 5.0 or pH 5.5 has been reported to induce acid tolerance in cultures grown at pH 7.0 (Rowbury and Hussain, 1998; Rowbury *et al.*, 1988). The low molecular weight protein identified was heat-stable and the possible products of the genes *hns*, *fur* or *himA* were implicated in forming the active component(s), since activity was almost inactive in filtrates prepared from strains with mutations in these genes (Rowbury and Hussain, 1998). It has been suggested that *E. coli* can signal acid tolerance to unadapted organisms through the secretion of a protein-signalling molecule (Rowbury and Hussain, 1998). Thus, this process of acid tolerance is unlike that of the classical acid tolerance response.

Acid resistance has been observed in stationary phase cells of *E. coli* using complex medium (Bearso *et al.*, 1997). This phenomenon is quite distinct from the ATR of *Salmonella* because cells are grown to exponential phase in defined minimal medium (Bearso *et al.*, 1997). In stationary phase, *E. coli* were found to be more acid resistant when challenged at pH 2.5 for a period of 2 hours in complex medium. No *Salmonella* isolates survived at pH 2.5, whereas, eighty percent of the *E. coli* strains tested were acid resistant (Gorden and Small, 1993; Bearso *et al.*, 1997). The type of medium and

growth conditions was important for the acid resistance of *E. coli* at an external pH of 2.5. No *E. coli* strains were shown to survive using minimal medium (Lin *et al.*, 1995). The acid resistance of *E. coli* was due to the presence of three stationary phase and low pH-inducible acid resistance systems. One was dependent on RpoS, and cAMP receptor protein-dependent system that protects cells at pH 2.0. Secondly, glutamate and the putative glutamate/gamma amino butyric acid antiporter (GadC) and thirdly the arginine-dependent system requiring arginine decarboxylase (AdiA) (Cheville *et al.*, 1996; Lin *et al.*, 1996; Ferreira *et al.*, 1999; Foster, 1999; Castanie-Cornet *et al.*, 1999).

1.12.2. *Aeromonas hydrophila.*

Aeromonas hydrophila is a waterborne pathogen and an enteric pathogen of man, therefore survival in low pH conditions is important to its persistence in the environment and for disease (Schubert, 1991). An acid tolerance response in *A. hydrophila* has been demonstrated with survival at extreme pH (Karem *et al.*, 1994). Cultures grown at pH 7.2 and adapted to pH 5.0 for 20 minutes before challenge at pH 3.5 exhibited higher survival than unadapted cultures. A log difference of greater than 5 was observed for cultures exposed to 1.5 hours post-acid challenge compared to the unadapted cultures (Karem *et al.*, 1994). The addition of chloramphenicol eliminated acid tolerance, indicating protein synthesis was required at the adaptive pH for subsequent survival to pH challenge (Karem *et al.*, 1994). An increase in the synthesis of 28 proteins and a decrease in 10 proteins was observed when cells were shifted to pH 5.0 (Karem *et al.*, 1994). The acid tolerance response in *A. hydrophila* was similar to that identified in *E. coli* (Raja *et al.*, 1991) and *S. typhimurium* (Foster, 1991).

1.12.3. *Listeria monocytogenes.*

Listeria monocytogenes is an important food-borne pathogen. Its ability to withstand environmental stresses, including low pH, plays a role in determining its survival as a pathogen in food and inside macrophages. Initial experiments demonstrated an induced acid tolerance in *Listeria monocytogenes* but with no explanation with regards to protein synthesis or systems involved (Kroll and Patchett, 1992). Further studies have demonstrated that during logarithmic growth of *L. monocytogenes*, a period of 60 minutes exposure at pH 5.0 prior to challenge at pH 3.0 resulted in maximal acid tolerance (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996). Full expression of the acid tolerance response required *de novo* protein synthesis, since the addition of

chloramphenicol resulted in substantial loss in acid tolerance (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996). Using two-dimensional gel electrophoresis, the expression of 53 proteins was shown to alter during acid adaptation (O'Driscoll *et al.*, 1997). Protein expression was also found to vary depending on the type of acid used, for example, lactic acid or hydrochloric acid (O'Driscoll *et al.*, 1997). Furthermore, acid-adapted cells demonstrated cross protection to other environmental stresses, such as thermal and osmotic stress (O'Driscoll *et al.*, 1996). A growth-phase-dependent acid tolerance system is also involved in *L. monocytogenes* that is independent of pH and is observed during the stationary phase (Davis *et al.*, 1996). It has been suggested that stationary phase cells use a different mechanism to that of the logarithmic grown cells to achieve acid tolerance (Datta and Benjamin, 1997).

1.12.4. Root Nodule Bacteria.

The effects of low pH on growth, survival, nodulation and nitrogen fixation by legume root nodule bacteria are important and have implications for legume production (Glenn and Dilworth, 1994). The growth rate of root nodule bacteria decreases as external pH falls therefore tolerance to acid would be beneficial for survival and nodulation by root nodule bacteria (Glenn and Dilworth, 1994). An acid tolerance response has been observed in strains of *Rhizobium* (O'Hara and Glenn, 1994). Cells transferred from pH 7.0 to pH 5.0 and subsequently exposed to pH 3.0, grew better than cells grown at pH 7.0 and exposed directly to pH 3.0. Protein synthesis was involved as chloramphenicol inhibited the acid tolerance response (O'Hara and Glenn, 1994). Cells grown to stationary phase were found to be more acid tolerant than logarithmically grown cells (O'Hara and Glenn, 1994). Using Tn5 mutagenesis, an acid sensitive mutant of *Rhizobium meliloti* WSM419 was found that failed to grow below pH 6.0 compared to the parent strain that could grow to pH 5.7 (Tiwari *et al.*, 1996). The DNA sequencing of the Tn5 insertion site identified two open reading frames, designated *actS* and *actR* that had high similarity to two-component sensor-regulator systems involved in signal transduction. The inactivation of *actS* or *actR* in *Rhizobium meliloti* resulted in acid-sensitive phenotypes, thus, implicating the ActS/ActR two-component system in acid tolerance in *Rhizobium meliloti* WSM419 (Tiwari *et al.*, 1996).

1.12.5. Propionibacteria.

Acidification is used as a means of preservation in the food industry to prevent spoilage by contaminating organisms. The use of organisms in the dairy and cheese industry result in exposure of the organisms to acidic pH. *Propionibacterium freudenreichii* is an important bacterium required for the production of Swiss-type cheeses and in *P. freudenreichii* subsp. *shermanii* strain the existence of an acid tolerance response has been demonstrated (Jan *et al.*, 2000). Optimal acid tolerance was observed when log-phase cells were pre-exposed to pH 4.5 for 30 minutes before acid challenge at pH 2. Stationary phase acid tolerance response was also demonstrated but these cells were more resistant than log phase cells, as seen in *Rhizobium* (O'Hara and Glenn, 1994; Jan *et al.*, 2000). In addition exposure to heat and starvation in log-phase cells resulted in partial protection towards acid, distinct from *Salmonella*, in which resistance to other stresses did not lead to acid tolerance (Lee *et al.*, 1995; Jan *et al.*, 2000).

Bacterial acid tolerance has resulted in contamination of products and has become a major problem to the food industry. An acid tolerant bacterium, *Propionibacterium cyclohexanicum* sp. nov., was isolated from spoiled orange juice (Kusano *et al.*, 1997) and was found to have 97.1% homology to *Propionibacterium freudenreichii*. Growth of this organism occurred at pH 3.2 to 7.5, with an optimum growth between pH 5.5 to 6.5. This bacterium produces a large amount of lactic acid, indicating that exceptionally effective adaptive mechanisms must exist in propionibacteria, which ensure their survival in extreme acidic environments (Kusano *et al.*, 1997). The mechanisms and involvement of genes or protein synthesis has not been demonstrated in either of these strains.

1.12.6. *Vibrio parahaemolyticus.*

Vibrio parahaemolyticus is a halophilic bacterium considered to be an important food-borne pathogen. The bacterium encounters a number of stresses, but in particular, pH stress is encountered during infection of the host (Wong *et al.*, 1998). In order to survive such stress, *V. parahaemolyticus* exhibits an acid tolerance response (Wong *et al.*, 1998). Log-phase cells grown at pH 7.5 and subsequently adapted at pH 5.0 for 30 minutes were more resistant to challenge by acid at pH 4.4 compared to unadapted cells. Adapting the cells to two different acidities, pH 5.8 followed by pH 5.0 for 30 minutes enhanced the acid tolerance of the organisms (Wong *et al.*, 1998). Although an acid

tolerance response was demonstrated, pH 4.4 was considerably higher than that employed to demonstrate acid tolerance in *Salmonella* (pH 3.3) (Foster and Hall, 1990). It has been suggested that due to the increased susceptibility of this bacterium to stress in the environment and *in vivo*, the acid tolerance response produced by mild exposure to lower pH could improve protection of *V. parahaemolyticus* against acid and other stresses. Also this would allow cross protection to other stresses (Wong *et al.*, 1998). Protein production was also shown to be involved in acid tolerance of *V. parahaemolyticus*; using one-dimensional polyacrylamide gel electrophoresis, 17 proteins were induced while 4 were inhibited. Using two-dimensional polyacrylamide gel electrophoresis an increased number of proteins were detected, 20 proteins were induced and 26 proteins were inhibited (Wong *et al.*, 1998).

1.13. Mycobacterial Survival and Growth in Conditions of Acid Stress.

Mycobacteria can encounter acid conditions in the environment and during an infectious process (Schulze-Robbecke, 1993). Acid conditions are generated in the environment by acid rain, pollution, sewage and decomposing vegetation. Free-living mycobacteria have been isolated from soil and water (Collins, 1984; Schulze-Robbecke, 1993). In particular *Mycobacterium chelonei* and *Mycobacterium sphagni* have been recovered from swamps, bogs and marshes in which sphagnum moss grows and are found to be particularly abundant in the decomposing stratum of the moss (Kadza, 1979). The fungal decomposition of the vegetation leads to release of nutrients such as amino acids (Stanford and Paul, 1973; Kadza, 1979) and overall, leads to the acidification of the environment.

Elsewhere low pH is a biologically relevant stress for mycobacteria since they encounter acidic environments during the infectious process. Mycobacteria can occur on skin, in the urinary tract and abdominal lesions where the pH is low (Grange, 1988). An acid pH has also been recorded in abscesses, pus and acid conditions may also arise during development of caseous necrosis. Areas of inflammation and caseation that develop in response to contact of tissue and tubercle bacilli can become markedly acidic, at least during certain stages of their development (Grange, 1988). The site of infection becomes acidic as fatty acids accumulate in the caseous matter of necrotic areas probably as a consequence of lipase activity during autolysis (Grange, 1988). In addition, increased production of lactic acid and other organic acids occurs in

metabolising tissue cells particularly in low oxygen conditions. It has been stated that they contain more lactic acid than do normal tissues (Dubos, 1950).

The antimycobacterial drug, pyrazinamide, is ineffective against *Mycobacterium tuberculosis in vitro* at neutral pH but maximal activity has been demonstrated under acidic pH conditions at pH 5.0 to 5.5 and the activity of pyrazinamide has also been demonstrated *in vivo* in tuberculous lungs of experimental animals (Tarshis and Weed, 1953; McDermott and Tompsett, 1954; McCune *et al.*, 1956). Decreasing numbers of *M. tuberculosis* in the presence of pyrazinamide is indicative that the bacteria are in an acidic environment (Heifets and Lindholm-Levy, 1992).

The success of mycobacteria as pathogens depends on their ability to maintain an infection inside the phagosome of the macrophage (Grange, 1988). Blocking the acidification of the host cell by preventing phagosome-lysosome fusion could bring about survival and infection of the host cell by *M. tuberculosis*. Thus, resulting in a decrease in hydrolytic enzyme activity and the impairment of the fusogenic activity of the organelles (Chicurel *et al.*, 1988). However, pH change is not only a consequence of phagosome-lysosome fusion but can arise independently in phagosomes prior to fusion (Grange, 1988). Acidity as low as pH 3.0 was demonstrated within the monocytes, where tubercle bacilli have been found to exist (McDermott and Tompsett, 1954). In addition, indicator dyes have been used to demonstrate that tubercle bacilli are situated in an acidic environment, when present in mouse and guinea pig peritoneal mononuclear phagocytes (Sprick, 1956).

Products of mycobacteria such as polyanions (Goren *et al.*, 1976) and ammonia (Gordon *et al.*, 1980) have been implicated as modulators of fusion between phagosome and lysosome. It has been demonstrated that macrophage lysosomal pH increased significantly after addition of culture filtrate of *M. tuberculosis* (Chicurel *et al.*, 1988). The filtrate containing mycobacterial antigens was able to inhibit lysosomal acidification possibly by interfering with essential lysosomal structures such as membrane permeability or H^+-ATPase pump. A second alkalinisation step is seen after the first pH has been restored suggesting a pH dependent mechanism is involved (Chicurel *et al.*, 1988). Further evidence for the role of H^+-ATPase in phagosomal acidification has been obtained. Vacuoles containing *Mycobacterium avium* failed to

acidify below pH 6.3 to 6.5, suggesting that fusion with proton-ATPase-containing vesicles was inhibited and thus prevented acidification of the cell (Sturgill-Koszycki *et al.*, 1994). Furthermore, vacuoles containing dead mycobacteria were found to be acidic, whereas vacuoles containing living mycobacteria were not, indicating that mycobacteria were able to prevent cell acidification (Crowle *et al.*, 1991; Barker *et al.*, 1997).

Strains of *M. tuberculosis* vary in sensitivity to low pH. Of 6 strains tested, survival of H37Ra, I2646, 79499 and 79112 were least affected by pH when exposed to various acidities for a period of ninety minutes. Two other strains, B1453 and 79112R showed a decrease in survival with decreasing pH (Jackett *et al.*, 1978). Not only have differences been observed between strains but variations have also been seen in slow and fast growing mycobacteria grown on acidic medium, although the sensitivity of mycobacteria was independent of culture medium and buffer used at the different pH values (Chapman and Bernard, 1962; Portaels and Pattyn, 1982). The slow growers prefer to grow in slightly acidic conditions compared with the fast growers (Portaels and Pattyn, 1982).

The hypothesis of the presence of an acid tolerance response in mycobacteria has arisen from studies in *Salmonella* and *E. coli*. An increasing number of acid regulated virulence factors are being identified some of which appear to function in intracellular survival. Initial experiments with *M. smegmatis* mc^2155 demonstrated an inducible acid tolerance response. Exposure of bacteria to an adaptive pH of 5.0 resulted in protection against subsequent challenge to a lethal pH of 3.5 using phosphoric acid or pH of 3.0 using hydrochloric acid. The acid tolerance response required *de novo* protein synthesis (O'Brien *et al.*, 1996). Consequently, adaptation of mycobacteria to acid may be an important part of the virulence of mycobacteria and hence represents an area of drug targeting.

1.14. Regulatory Genes Involved in Acid Tolerance of *Salmonella typhimurium*.

The acid tolerance response enables *Salmonella typhimurium* to survive exposure to potentially lethal acidic environments. In *S. typhimurium* approximately fifty acid shock proteins have been identified that are synthesised in response to acid stress (Foster 1995). Some of these acid shock proteins are regulated by regulatory genes (Foster,

1995). Several regulatory genes have been found to play a role in the log phase acid tolerance response, these include *rpoS* that codes for the alternative sigma factor, *fur* that codes for the iron regulator and *phoPQ* that codes for a two-component signal transduction system. Each regulator controls the expression of a subset of acid shock proteins (Foster, 1999).

1.14.1. The Sigma Factor.

The alternative Sigma Factor (σ^S) is encoded by *rpoS* and has been implicated in regulating a feature of acid tolerance. The σ^S-dependent acid tolerance response system is required for surviving volatile fatty acids and contributing to organic acid stress (Foster, 1999). During log phase growth at pH 7.7, the σ^S is turned-over rapidly via proteolysis by ClpXP protease and is therefore scarcely detected, however, acid shock induces σ^S (Foster, 1999). MviA, a 38-kDa protein encoded by the mouse virulence gene *mviA*, has been implicated in controlling the induction of σ^S during acid shock. The N-terminal domain of MviA shows sequence homology to the regulatory proteins of two-component signal transduction systems (Bearson *et al.*, 1996). MviA, identified as a response regulator, acts by controlling the accumulation of σ^S by signalling decreased proteolytic turnover and in turn allows the levels of σ^S to increase in response to acid shock (Lee *et al.*, 1995; Bearson *et al.*, 1996; Foster, 1999). MviA was shown to stimulate σ^S turnover in the absence of stress but allowed its accumulation in the presence of stress (Bearson *et al.*, 1996). Mutations in *mviA* (*mviA*::Km) resulted in overproduction of σ^S and σ^S-dependent acid shock proteins in cells grown logarithmically and also resulted in elevated levels of acid tolerance, resistance to heat, oxidative stress and osmolarity. Mutations in *rpo*S suppressed these *mviA*::Km associated defects, suggesting that the effects of MviA on cell physiology occurred via its control of σ^S levels (Bearson *et al.*, 1996).

The sustained induction system generated in the acid tolerance response when triggered at log phase by acid shock is dependent on RpoS (Lee *et al*, 1995). The transient system requiring Fur and AtrB are RpoS-independent. Western blot analysis and two-dimensional polyacrylamide gel electrophoresis revealed that RpoS was an acid shock protein that induced a further eight acid shock proteins (Lee *et al*, 1995). In addition, the sustained ATR was cross protective to other environmental stresses but adaptation to other stresses did not protect against acid tolerance (Lee *et al*, 1995). Thus, the acid

tolerance response systems are independent of each other (Lee *et al*, 1995). However AtrB (fatty acid synthesis) has been identified as involving the two ATR systems and has been found to be negatively regulated by the regulator AtbR and σ^S. Mutations in the *atbR* gene resulted in over-expression of these proteins and conferred a constitutive acid tolerance phenotype (Lee *et al.*, 1995). These mutations were specific to log phase ATR and did not effect stationary phase ATR (Lee *et al.*, 1995).

The role of RpoS in the virulence of *Salmonella* has been investigated (Fang *et al.*, 1992; Lee *et al.*, 1995; Wilmes-Riesenberg *et al.*, 1997). Studies have demonstrated that virulent strains of *Salmonella* exhibited higher acid tolerance than LT2, an avirulent laboratory strain. This phenomenon was attributed to a mutation identified in the *rpoS* allele of LT2. In addition, mutations in *rpo*S rendered *Salmonella* avirulent (Fang *et al.*, 1992; Lee *et al.*, 1995; Wilmes-Riesenberg *et al.*, 1997). Mutations in *mviA* also caused *Salmonella* to become avirulent suggesting that the organism must be able to freely change the response of σ^S (Benjamin *et al.*, 1991).

1.14.2. Iron Regulator Fur.

The 17-kDa Fur protein controls a series of genes that are involved in synthesis, excretion and recovery of the iron-chelating siderophore enterochelin (Visca *et al.*, 1991). Regulation of iron uptake is necessary for protection of the cell since high intracellular concentrations of iron can be toxic. During periods of high internal Fe(II) concentrations, the iron-Fur complex binds to the Fur box, a 19 base pair DNA consensus sequence, in the promoter region of the iron acquisition genes in order to repress their transcription (Bagg and Neilands, 1987).

The iron regulator Fur is required for acid tolerance and it is induced by acid (Hall and Foster, 1996). Verification of these findings arose from mutagenesis studies of *fur*. Mutants of *fur* displayed an acid-sensitive phenotype; cells were extremely sensitive to pH 3 when previously adapted at pH 5.8 for one doubling. An *E. coli fur*-containing plasmid restored acid tolerance in *S. typhimurium* proving that Fur was essential for the acid tolerance response (Hall and Foster, 1996). Random mutagenesis studies also indicated that acid tolerance and iron-regulatory mechanisms of *S. typhimurium* are sensed separately. A mutation converting histidine 90 to an arginine (H90R) in the Fur sequence diminished Fur-mediated iron regulation of enterochelin production but had

no affect on the production of acid shock proteins (Hall and Foster, 1996). This suggested that this histidine residue was important for iron regulation but not for the regulation of acid tolerance. Nine acid shock proteins were identified as being regulated by Fur in an iron-independent approach (Hall and Foster, 1996).

1.14.3. PhoPQ Two Component System.

The two-component regulatory system PhoP and PhoQ has proved to be important for tolerance to inorganic acid stress and is regulated in an RpoS-independent manner (Bearson *et al.*, 1998). The PhoPQ regulon autoregulates its own expression (Soncini *et al*, 1995) and is known to be important in growth within macrophages, protection against antimicrobial peptides and virulence (Fields *et al.*, 1986; Fields *et al.*, 1989; Belden and Miller, 1994; Gunn and Miller, 1996; Mahan *et al.*, 1996).

PhoQ is an inner membrane sensor-kinase protein that functions to promote survival of *S. typhimurium* within macrophages. PhoP is a 26-kDa protein that functions as a transcriptional activator and has also been identified as an acid shock protein (ASP29) (Foster, 1991). Specifically, *phoP* mutants failed to induce four acid shock proteins confirming a role for this regulator in acid tolerance (Bearson *et al.*, 1998). Further evidence demonstrates that PhoPQ dependent systems protect against inorganic but not organic acid stress. A *phoP* mutant was tested for inorganic acid tolerance and was found to be extremely sensitive compared to a *phoP* positive cell. The inorganic acid used in the study was not stated. Additionally, both *phoP* and *phoQ* were required for acid tolerance, since mutations in any one of these genes or both resulted in acid sensitive phenotypes (Bearson *et al.*, 1998).

The current model for PhoPQ regulation proposed that PhoQ undergoes conformational change upon sensing extracytoplasmic magnesium and was not considered to be a pH sensor (Soncini and Groisman, 1996). Under high magnesium concentrations, PhoQ was inhibited and did not phosphorylate PhoP, so that PhoP was not active as a DNA-binding protein (Soncini and Groisman, 1996). Furthermore, it was suggested that the pH control of a subset of PhoP-regulated genes involved the *pmrCAB* operon that encoded a two-component regulatory system (mediates resistance to polymyxin B) (Roland *et al.*, 1993; Gunn and Miller, 1996), which itself could be activated by PhoP (Soncini and Groisman, 1996). The response regulator PmrA is like PhoP, a positive

transcription factor. PmrB is a sensor kinase that senses extracellar pH and has been identified as a transmembrane protein. A decrease in pH is seen to cause PmrB autophosphorylation which subsequently proceeds to phosphorylate PmrA. The role of PmrC is unknown but has been identified as a putative membrane protein (Soncini and Groisman, 1996). Overall, the model proposed that genes dependent on PhoP were controlled separately by pH and magnesium (Soncini and Groisman, 1996).

This model was reinvestigated to verify these findings (Bearson *et al.*, 1998). If the model was correct and since PhoP was shown not to sense pH, loss of PhoP should abolish the magnesium control of these genes but not their regulation by pH. Equally, a *pmrA* mutation should abolish pH control but not regulation by magnesium. To test this model, a *pagA-lacZ* fusion was constructed for use as a reporter of PhoP activity since *pagA* induction is PhoP-dependent (Bearson *et al.*, 1998). Acidic pH was found to induce *pagA* in a PhoP-dependent manner even in the presence of high concentrations of magnesium that would normally repress PhoP. In addition, when a *pmrA* mutation was tested, it was found to be essential for the acid induction of *pagA-lacZ* at high concentrations of magnesium, as well as for the low magnesium response and thus demonstrated that both PhoPQ and PmrAB appeared to sense acid stress (Bearson *et al.*, 1998).

In view of these current findings a further model that attempts to combine magnesium, H^+, PhoPQ and PmrAB in regulating gene expression has been proposed (Bearson *et al.*, 1998). It was suggested that there were two groups of genes regulated by PhoP; one group of genes required PhoP-phosphate (PhoP-P) for induction and which were PmrA-independent and the other group required PhoP-P and were dependent on PmrA-phosphate (PmrA-P). The different levels of magnesium sensed by PhoQ would subsequently result in attainment of different levels of PhoP-P that would ultimately affect the response of the first group of genes. However, the amount of H^+ levels would also influence the levels of magnesium required to produce a given level of PhoP-P. Thus, genes that required low quantities of PhoP-P for induction could respond to either low magnesium or low pH. Genes that required high quantities of PhoP-P for induction would be induced under lower levels of magnesium at neutral pH or under more moderate magnesium levels in an acidic environment. In addition, a PhoP-PmrA cascade that amplified the pH response was suggested in regulating the second group of

genes. Specifically, under acidic environments (even under high magnesium conditions) adequate PhoP-P could be produced leading to induction of the PmrA system. The PmrA system would subsequently undergo autoinduction that would arise to amplify the pH signal. The ensuing high levels of PmrA-P generated either through the PmrB sensor kinase sensing pH directly or through some other signal (a second promoter) would then fully induce the PmrA subset of PhoP-regulated genes (Bearson *et al.*, 1998).

The involvement of magnesium is further demonstrated in magnesium transport in *S. typhimurium*. PhoPQ controls the expression of several genes and two of these include *mgtA* and *mgtCB* that codes for magnesium uptake systems in *S. typhimurium* (Soncini *et al.*, 1996; Moncrief and Maguire, 1998). MgtA and MgtB are P-type ATPases (Snavely *et al.*, 1991; Tao *et al*, 1995; Soncini *et al.*, 1996). Two stages occur in response to magnesium deficiency, the first stage leads to a several hundred-fold increase in expression of *mgtA* and *mgtB* to increase internal magnesium. The second stage occurs if magnesium levels do not increase within six to ten hours, expression of *mgtA* increases 10,000-fold and expression of *mgtB* increases 2000-fold (Soncini *et al.*, 1996). An efficient PhoPQ system was required for both stages of MgtB activity but MgtA was only dependent on PhoPQ in the first stage and therefore a second mechanism may be involved in the second stage (Soncini *et al.*, 1996). MgtC is not a magnesium transporter and does not play a role in survival of *S. typhimurium* at low pH. An *mtgC* positive strain failed to grow at pH 5.2 though MgtC was produced, indicating that expression of *mgtC* by itself did not confer survival at low pH (Moncrief and Maguire, 1998). However, MgtC was shown to be essential for long-term survival within macrophages (Moncrief and Maguire, 1998).

The induction of PhoPQ by low pH also has important implications for virulence (Bearson *et al.*, 1998). Survival within phagosomes requires five *pag* genes that are regulated by PhoP and PhoQ proteins. The *pag* genes in *S. typhimurium* are PhoP-activated and have been identified as virulence factors (Aranda *et al.*, 1992). Three of these genes exhibited increased expression within macrophages upon acidification of the vacuoles, *pagA*, *pagB* and *pagC* and thus suggested that pH was one factor to elicit PhoP-mediated activation (Olson, 1993). Subsequently, addition of weak bases, ammonium chloride and chloroquine prevented internal acidification and as a result PhoP-regulated gene expression was abolished preventing transcription of *pag* genes

(Aranda *et al.*, 1992). In addition, bacterial phagocytosis by murine macrophages resulted in 50- to 77-fold increase in PhoP-activated gene expression but no activation was found in epithelial cells suggesting that PhoQ signals were specific to macrophage phagosomes (Aranda *et al.*, 1992). Contradictory to this finding was the report that activation of *in vivo*-induced genes including *phoP* by PhoPQ was induced upon entry into and growth within human epithelial cells (Heithoff *et al.*, 1999). Behlau and Miller suggested that survival of *Salmonella* within epithelial cells required proteins encoding *phoP*-repressed genes and therefore virulence within epithelial cells and macrophages was modulated oppositely by the PhoPQ virulence regulators (Behlau and Miller, 1993). Further characterisation of PhoP led to the identification of thirteen new PhoP-activated virulence loci (Belden and Miller, 1994). In addition, the ability of viable *Salmonella* to delay and reduce phagosome acidification suggests that survival is partly due to maintaining a high pH (Aranda *et al.*, 1992). Phagosomes containing heat-killed *S. typhimurium* attained a pH of less than 4.5 in 1 hour compared to 4 to 5 hours to attain a pH of less than 5.0 using viable bacteria (Aranda *et al.*, 1992).

Use of *in vivo* expression technology (IVET) has led to the identification of additional bacterial genes induced during infection (Heithoff *et al.*, 1999). The expression levels of *in vivo*-induced (*ivi*) genes were increased in host tissue. Eight genes were identified that responded to low pH and low magnesium and three genes were identified that responded to iron. PhoP was found to regulate all eight genes; seven of these genes (*mgtA*, *mgtB*, *spvB*, *phoP*, *prmB*, *iviVI-A* and *iviXVI*) were PhoP-activated genes (*pags*) and their expression was dependent on a fully functional PhoP protein. The level of expression of the *iviXVII* (*pdu*) gene was increased in the absence of PhoP and was therefore named the PhoP-repressed gene (*prg*). Mutations in *phoQ* caused the PhoQ protein to become less sensitive to magnesium, leading to enhanced phosphorylation of PhoP and increased expression of *pags* and reduced expression of *prgs* compared to the wild type (Heithoff *et al.*, 1999).

1.15. Mechanisms of pH Homeostasis.

The maintenance of a relatively constant internal pH over a broad range of external pH is a process known as pH homeostasis. External changes in pH are sensed by bacteria and they respond accordingly, by adjusting the activity and synthesis of proteins associated with many different processes (Olson, 1993). Regulation of internal pH is

achieved through three main mechanisms, cytoplasmic buffering, proton transport and production of acid and bases in the cytoplasm (Booth, 1985).

1.15.1. Cytoplasmic Buffering.

Cytoplasmic buffering can only counterbalance limited changes in acidification or alkalinisation of the cytoplasm. The amino acid side chains of proteins have been implicated as the main components in the buffering of the cytoplasm (Booth, 1985). The internal buffering capacity of organisms varies between bacterial species. *Bacillus subtilis* was shown to attain a very high cytoplasmic buffering capacity in the acidic pH range compared to other species, for example, *Escherichia coli* (Krulwich *et al.*, 1985). In a separate study, similar values for cytoplasmic buffering capacity were found in *E. coli* over the pH range 5.0 to 7.5 (Rius *et al.*, 1995). Loss of pH homeostasis active mechanisms result in instant alkalinisation of the cytoplasm and no protection of the internal pH is offered from cytoplasmic buffering (Krulwich *et al.*, 1985). Inactivation of the Na^+/H^+ antiporter of *Bacillus firmus* resulted in an increase in cytoplasmic pH. Thus, the high buffering capacity found in *Bacillus* could indicate a possible mechanism for the protection of specific macromolecules or processes (Krulwich *et al.*, 1985).

Studies have found that buffering capacity increases with changes in internal pH (Zychlinsky and Matin, 1983). Proton influxes were measured in cells treated with inhibitors of respiratory pumps and ionophores. A strong buffering capacity was demonstrated in *Thiobacillus acidophilus* cells in which the buffering capacity increased with a decreasing pH from pH 5 to pH 4 (Zychlinsky and Matin, 1983). The influx of protons from actively respiring cells was immediately expelled through the respiratory pump. The addition of respiratory pump inhibitors prevented proton removal out of the cells and led to continued proton influx resulting in accumulation of protons. The cytoplasmic pH was not significantly affected by the influx of protons activated during metabolic inert conditions due to the sufficient buffering capacity of the cytoplasm (Zychlinsky and Matin, 1983). Further evidence in support of these findings came from a study which found that the acidophile PW2 could maintain a large pH gradient, even in the presence of proton pump inhibitors (Goulbourne *et al.*, 1986). This demonstrated the efficiency of the homeostasis system in these organisms.

Overall, an outwardly directed membrane potential must be generated in acidophiles at extremely acidic pH, such that a large pH gradient is maintained in these organisms allowing internal pH to remain near neutrality (Matin, 1999).

1.15.2. Proton Transport.

Proton transport is a key mechanism by which cytoplasmic pH is regulated by the movement of protons across the membrane. Protons are transported into and out of the cell via three major systems, the antiport system that exchanges ions such as K^+, Na^+ and H^+, proton-translocating ATPase (H^+-ATPases) that combine proton movement with the synthesis and hydrolysis of ATP and electron transport chains that are responsible for H^+ efflux (Olson, 1993).

1.15.2.1. Antiport System.

Sodium and potassium antiports have been suggested to play a dominant role in the acidification of the cytoplasm (Booth, 1985). The role of the antiporter is to acidify the cytoplasm when cells are grown in alkaline conditions in order to regulate internal pH and therefore the antiporter is activated by alkaline rather than acid pH (Olson, 1993). Internal sodium or potassium in the cell is exchanged, via the antiporter, for external protons, resulting in the acidification of the cytoplasm, but may act adversely to change internal pH if the load on internal pH causes the cytoplasmic pH to fall (Booth, 1985). The majority of work done on the antiport systems in bacteria has concentrated mainly on high pH systems. Cation-proton antiporter activity has been found in *Bacillus alcalophilus* (Krulwich *et al.*, 1986), *Bacillus subtilis* (Ito *et al.*, 1999) and *Saccharomyces cerevisiae* (Ros *et al.*, 1998).

The most recognised sodium-proton antiporter for sodium expulsion in *E. coli* is NhaA. The *nhaA* (*ant*) gene encodes for this sodium-proton antiporter (Goldberg *et al.*, 1987). However, two antiporters are involved NhaA and NhaB. Using β-galactosidase activity to study regulation of the expression of an *nhaA* and *lacZ* fusion (*nhaA'-lacZ'*), it was demonstrated that expression of *nha*A was regulated by *de novo* protein synthesis and pH. Cells at pH 7.5 and exposed to 60 mM-100 mM sodium chloride or lithium chloride demonstrated an increase of 5 to 10-fold in the activity of β-galactosidase. However, at pH 8.6 a similar response in activity was observed using only 10 mM sodium chloride or lithium chloride. Thus sodium and lithium ions have been implicated as inducers of

the *nhaA* gene and *nhaA* expression system is pH dependent (Karpel *et al.*, 1991). In *E. coli*, NhaB is a pH independent Na^+/H^+ antiporter that confers no dependence on pH in the range 6.4 to 8.3 (Padan *et al.*, 1989). NhaB is not essential for sodium tolerance since NhaA alone will confer complete tolerance. However, NhaB is required for sodium tolerance when NhaA activity is growth limited or when NhaA is not activated or when *nhaA* is not adequately expressed (Pinner *et al.*, 1993).

In *E. coli* a positive regulator of *nhaA* has been identified, NhaR, that was homologous to a family of bacterial transcriptional regulatory proteins LysR-OxyR. An *nhaR*-containing plasmid enhanced the expression of an *nhaA′-lacZ′* fusion (Rahav-Manor *et al.*, 1992). A deletion mutant of *nhaR* (OR100) established that *nhaR* was required, in addition to *nhaA*, to tolerate extreme conditions. However, findings from *nhaA* mutant strains implied that the regulation exerted by *nhaR* was incomplete and some expression of *nhaA* still existed, as *nhaR* mutants were more resistant to sodium ions and alkaline pH than *nhaA* mutant strains (Rahav-Manor *et al.*, 1992). Furthermore, NhaR has been identified as a sensor and transducer of the sodium signal that regulates expression of *nhaA* by undergoing a conformational change upon binding sodium, with this occurring in a pH-dependent manner (Carmel *et al.*, 1997).

Potassium transport systems have also been implicated in playing a role in pH homeostasis in the acid external pH range yet the details of the proposed mechanisms are unclear (Padan and Schuldiner, 1986). Potassium plays an important role in bacterial growth and in the physiology of the cell (Kakinuma, 1998). Studies have been done in *E. coli*, *Salmonella typhimurium* and the Gram-positive bacterium, *Enterococcus hirae* to investigate regulation of cytoplasmic pH in these organisms via the K^+/H^+ antiport system to demonstrate the role of this system in the control of internal pH (Bakker and Harold, 1980; Kobayashi, 1982; Bakker *et al.*, 1987; Epstein *et al.*, 1993; Kakinuma and Igarashi, 1988; 1999).

The role of the K^+/H^+ antiporter has been studied in the marine bacterium, *Vibrio alginolyticus* (Nakamura *et al.*, 1984). The acidification of the cytoplasm at alkaline pH is driven by the K^+/H^+ antiporter, potassium is driven in an outwardly direction and protons enter the cell causing an inside acidic pH gradient. The antiporter ceases to function when an internal pH of 7.8 is reached. This internal pH is regulated over the

pH range 6.0 to 9.0 and potassium is essential for regulation (Nakamura *et al.*, 1984). The addition of potassium chloride to the external medium at the acidic pH range is essential since the entry of K^+, would allow more H^+ to be removed from the cell by the primary proton pumps allowing for internal alkalinisation. Overall, this study demonstrated that the activity of the K^+/H^+ antiporter is dependent on the internal pH (Nakamura *et al.*, 1984).

The K^+/H^+ antiport system, Na^+-ATPase and Na^+/H^+ antiporter were all found to be unnecessary for growth of *Enterococcus hirae* at low pH (Kakinuma *et al.*, 1999: Ikegami *et al.*, 2000). The K^+/H^+ antiport system was absent in *E. hirae* cultures grown in defined medium, however cultures could grow below pH 7.5 but did not grow very well at pH 9 (Kakinuma *et al.*, 1999). Furthermore, a mutant deficient in Na^+-ATPase and Na^+/H^+ antiporter activity could grow at pH 5.5 in the presence of high sodium and demonstrated normal intracellular levels of sodium and potassium. However, the mutant was unable to grow above pH 7.5 in high sodium levels (Ikegami *et al.*, 2000). Thus, demonstrating that these systems are not necessary for growth at low pH but are required at high pH.

1.15.2.2. Proton-Translocating ATPase (H^+-ATPase).

The proton-translocating ATPase (H^+-ATPase) is an enzyme complex in the membrane of cells that carries out the synthesis of ATP. A proton motive force is generated consisting of a pH gradient and transmembrane potential. Mitchell (1979) postulated that the driving force for the synthesis of ATP by the H^+-ATPase complex is the proton-motive force. Thus, the primary energy-conserving event in this model is the movement of protons across the cytoplasmic membrane (Stryer, 1988). The role of H^+-ATPase in conferring acid tolerance has been acknowledged in lactococcal, enterococcal and streptococcal species (Olson, 1993).

The regulation of pH in the cytoplasm is brought about by the expulsion of protons from the cell via the F_0F_1–ATPase complex with the concurrent uptake of potassium through its transport system, in relation to a decrease in internal pH. The mechanism involves the coupling of proton movement with the synthesis and hydrolysis of ATP (Stryer, 1988). It has been proposed that the *Enterococcus hirae* (previously known as *Streptococcus faecalis*) proton-translocating ATPase (H^+-ATPase) is a typical F_0F_1–

ATPase, however its function is different from other F_0F_1–ATPases (Shibata, 1992). In *E. hirae*, its role is to maintain a neutral cytoplasmic pH rather than one of synthesising ATP (Shibata, 1992).

Since enterococci have no respiratory chain, the proton motive force is generated by means of the H^+-ATPase. In fact, the level of H^+-ATPase of *E. hirae* increased as cytoplasmic pH was lowered to less than pH 7.6 to allow alkalinisation of the cytoplasm. This increase in H^+-ATPase was independent of the proton motive force and increased in the presence of protonophores, indicating control of pH and the ability to grow at low external pH (Kobayashi *et al.*, 1984). Further evidence suggested that the cytoplasmic pH of *E. hirae* is only regulated by H^+-ATPase, as mutants defective in H^+-ATPase showed an inability to alkalinise the cytoplasm (Kobayashi, 1985). No acidification system has been identified in *E. hirae* for the regulation of cytoplasmic pH at alkaline pH. The lack of acidification system has been attributed to the production of lactate during metabolism leading to the subsequent lowering of pH of the cytoplasm and surrounding medium. This may explain why an acidification system in *E. hirae* is not crucial for bacterial viability (Kobayashi, 1985).

The activity of H^+-ATPases in ruminal bacteria has also been assessed. *Streptococcus bovis* and *Megaspaera elsdenii*, two acid-tolerant bacteria, produced more H^+-ATPase than *Ruminococcus albus* and *Fibrobacter succinogenes*, two acid-intolerant bacteria (Miwa, 1997). The levels of H^+-ATPases increased more than two-fold when the acid-tolerant bacteria were grown at the lowest pH allowing for growth, whereas little increase in H^+-ATPases was reported in the acid-intolerant bacteria, even when grown at neutral pH (Miwa, 1997). This implies that the amount of functional H^+-ATPase determines bacterial susceptibility to acid.

Lactococci are known to possess an inducible acid tolerance response that involves the synthesis of proteins. The relationship between the ATR and H^+-ATPase in *Lactococcus lactis* was observed (O'Sullivan and Condon, 1999). High levels of H^+-ATPase resulted in high levels of acid tolerance in cells, in response to a decrease in internal pH. The increase in the levels of H^+-ATPase with decreasing internal pH has led to the implication that H^+-ATPase is an ATR protein, whose synthesis increases in *L. lactis* in response to an acidic internal pH (O'Sullivan and Condon, 1999).

Further evidence for the role of the proton-translocating ATPase in ATR comes from genetic studies of *Salmonella typhimurium*. The H^+-ATPase is encoded by the *atp* operon of *S. typhimurium* and may play a role in the inducible pH homeostasis system, enabling cells to maintain a higher internal pH during extreme acid exposure, after an adaptation period. Mutants of the *atp* gene, lacking ATPase, have acid sensitive phenotypes and do not survive even though they have been adapted to an initial mild acidic pH (Portillo *et al.*, 1993). This is further evident from the addition of inhibitors of ATPase activity that resulted in prevention of proton translocation and a loss of the ATR (Foster and Hall, 1991). This suggests that the vital role of the proton-translocating ATPase during the acid tolerance response is the expulsion of protons from the cell (Foster and Hall, 1991; Portillo *et al.*, 1993). The virulence of *atp* mutants also diminished suggesting that the H^+-ATPase is required for virulence in *S. typhimurium* (Portillo *et al.*, 1993).

1.15.2.3. Electron Transport Chain.

In an electron transport chain, electrons are transferred from NADH to oxygen through a chain of protein complexes. Electron flow across the cytoplasmic membrane, through these complexes, leads to the expulsion of protons across the membrane. The abundance of some of the proteins involved has been found to vary as a function of pH (Olson, 1993).

The role of cytochromes in electron transport chains has been cited as being important in relation to pH under aerobic and anaerobic conditions. Two operons present in *Escherichia coli*, *cyoABCDE* and *cydAB* catalyse the oxidation of ubiquinol-8 and reduction of water and oxygen. *cyoABCDE* encodes for cytochrome *o* oxidase (*cyo*), and under levels of low oxygen the low-affinity *cyo* is repressed and the high affinity cytochrome *d* system (*cyd*-encoded by *cydAB*) is induced (Cotter *et al.*, 1990). Two regulators, Fnr - fumarate and nitrate reductase regulation - (Unden and Schirawski, 1997) and ArcA - aerobic respiratory control - are involved in the repression of *cyo* (Cotter and Gunsalus, 1992).

The effect of pH was demonstrated through the expression of *cyoA'-'lacZ* and *cydA'-'lacZ* fusions in *E. coli* (Cotter *et al.*, 1990). Under aerobic conditions, *cyoA'-'lacZ*, which is normally induced under these conditions, varied fourfold as external pH

increased from pH 5.5 to pH 7.5; the expression of *cydA'-'lacZ* was unaffected by these conditions. The pH effect was independent of *fnr* (Cotter *et al.*, 1990). During anaerobic growth, gene expression was completely repressed in an fnr^+ strain at pH 5.5 to 7.5. However, in *fnr* deletion strains, under anaerobic conditions *cyoA'-'lacZ* expression increased 40-fold as the pH increased from pH 5.5 to pH 7.5. The expression of *cydA'-'lacZ* also decreased 6-fold in the *fnr* deletion strains as the pH increased from pH 5.5 to 7.5. Maximal repression was seen below pH 6.0, in an *fnr* deletion strain, under anaerobic conditions. Thus, Fnr functions better at low pH and acts as a repressor of gene expression in response to anaerobiasis. Overall, the expression of *cyo* prevails at high pH in aerobic conditions, where external acidification enhances growth, whereas *cyd* expression prevails at low pH in anaerobic conditions, where external acidification needs to be minimised (Cotter *et al.*, 1990).

Furthermore, under anaerobic conditions, Fnr also functions as a repressor of *cydAB* expression. However, since expression of *cydAB* was four times higher than the wild type in an *fnr* deletion strain, a second regulatory gene as been implicated in controlling gene expression (Cotter *et al.*, 1990). The ArcA of the two-component regulatory system, ArcA/ArcB functions to activate *cydAB* gene expression under oxygen limiting conditions whilst Fnr represses expression (Cotter and Gunsalus, 1992; Tseng *et al.*, 1996; Cotter *et al.*, 1997).

Archaea are the most extremophilic of the acidophilic bacteria that can grow at pHs between 1 to 3 and yet they are able to maintain a near neutral internal pH (Schäfer, 1999). In acidophiles the extrusion of protons from the cytosol is mainly through primary proton pumps that are energised by respiratory redox systems. The SoxABCD complex of *Sulfolobus acidocaldarius* is thought to actively pump protons by H^+/O (proton/oxygen translocation) through an aerobic electron transport chain. However, *S. acidocaldarius* does not have a typical respiratory chain. No c-type cytochromes are known to exist in this bacterium and thus it relies on terminal oxidase complexes in proton pumping. Thus, atypical systems are involved in the regulation of internal pH in archaea (Schäfer, 1999).

1.15.3. Inducible pH Homeostasis - Amino Acid Decarboxylases.

Amino acid decarboxylases are involved in pH homeostasis and two types of decarboxylases exist in *Escherichia coli*; the constitutive biosynthetic and inducible biodegradative decarboxylases. The constitutive biosynthetic enzymes consist of arginine decarboxylase (*speA*), ornithine decarboxylase (*speC*) and diaminopimelic acid decarboxylase (*lysA*) (Tabor and Tabor, 1985). They are involved in the synthesis of polyamines that are expressed at low levels independently of pH. The inducible biodegradative enzymes consist of arginine decarboxylase (*adi*), glutamic acid decarboxylase (*gadS*), histidine decarboxylase, ornithine decarboxylase (*dcor*) and lysine decarboxylase (*cadA*) (Gale, 1946). The inducible biodegradative enzymes produce basic amines in response to low pH and thus are presumed to play a role in maintaining pH homeostasis or detoxifying the external medium as a protective mechanism for bacteria to survive and grow in acidic pH conditions.

The best-characterised system of response to external acid is that of the amino acid decarboxylase, lysine decarboxylase. The induction and regulation of lysine decarboxylase has been studied extensively in *E. coli*. Lysine decarboxylase is encoded by *cadA* and situated immediately upstream of *cadA* is *cadB*; both genes form an operon that have been mapped at minute 93.5 on the *E. coli* chromosome (Tabor *et al.*, 1980; Auger *et al.*, 1989; Watson *et al.*, 1992; Meng and Bennett, 1992a). Expression of *cadA* is maximally induced by low pH, excess lysine and anaerobiasis (Auger *et al.*, 1989). Lysine decarboxylase consists of a polypeptide of 715 amino acids and CadB consists of a polypeptide of 444 amino acids (Meng and Bennett, 1992a; Watson *et al.*, 1992). It has been proposed that CadB is a lysine/cadaverine antiporter. The characteristics of CadB are similar to that found for other membrane proteins and is highly hydrophobic, which suggests that CadB is a membrane-associated protein. In addition, the CadB sequence has homology to the ArcD protein of *Pseudomonas aeruginosa*, encoding an arginine/ornithine antiporter (Meng and Bennett, 1992a).

The promoter, P*cad*, has been identified upstream of *cadB* and is responsible for pH regulation of *cadA* expression in *E. coli* (Watson *et al.*, 1992). The sequence upstream of *cadB* revealed the presence of a hexanucleotide similar to the consensus hexanucleotide sequence found in *E. coli* σ^{70}-dependent promoters. Mutations introduced into the -10 and -35 hexanucleotide sequences using site-directed

mutagenesis and cloned upstream of *lacZ* resulted in the loss of pH-induced *lacZ* expression (Watson *et al.*, 1992). Further evidence from Northern hybridisation and primer extension experiments indicated the presence of a promoter responsible for the regulation of *cadA* and *cadB* expression (Watson *et al.*, 1992). Another open reading frame upstream of P*cad* has been identified as *cadC* and codes for a transcriptional activator necessary for pH-induced expression of P*cad* (Watson *et al.*, 1992). Furthermore, the carboxy terminus of CadC detects lysine as a positive regulator and cadaverine as a negative regulator (Dell *et al.*, 1994; Neely *et al.*, 1994).

A proposal has been made for the mechanism of action of amino acid decarboxylases in the growth of bacteria under acidic conditions and the ability to detoxify external acidic pH (Figure 1.3.) (Meng and Bennett, 1992a; Watson *et al*, 1992). At low external pH, the *cadBA* operon is induced and leads to the production of lysine decarboxylase in the cells of *E. coli*. The lysine/cadaverine antiporter, CadB, transports lysine into the cell which in turn becomes decarboxylated by lysine decarboxylase. The alkaline end-product that is formed, cadaverine, is excreted into the external medium, thus increasing the external pH. Permeability of the cell to protons increases at low pH and protons may also be taken into the cell via the CadB transporter from the external medium and utilised for lysine decarboxylation thus decreasing proton levels in the external medium (Meng and Bennett, 1992a). The lysine decarboxylase reaction could eliminate a large quantity of protons from the cell; one proton is utilised per molecule of lysine processed. However, in order to raise the external pH, it has been suggested that an inward gradient of H^+ could occur at the same time as lysine uptake via the CadB system. Although this inward gradient would lead to a higher internal H^+ concentration, the slight raise in external pH could benefit the culture and allow extended growth. Overall, an inward gradient would be created allowing lysine and protons to enter the cell and an outward gradient allowing cadaverine and carbon dioxide to leave (Meng and Bennett, 1992a).

In view of the large amounts of lysine present in the medium and induction of the *cad* operon, a substantial amount of cadaverine would be formed. However no pathways to metabolise cadaverine in *E. coli* are known. In fact, high levels of polyamines can be toxic to the cell and prevent functioning of the lysine decarboxylase. Consequently it

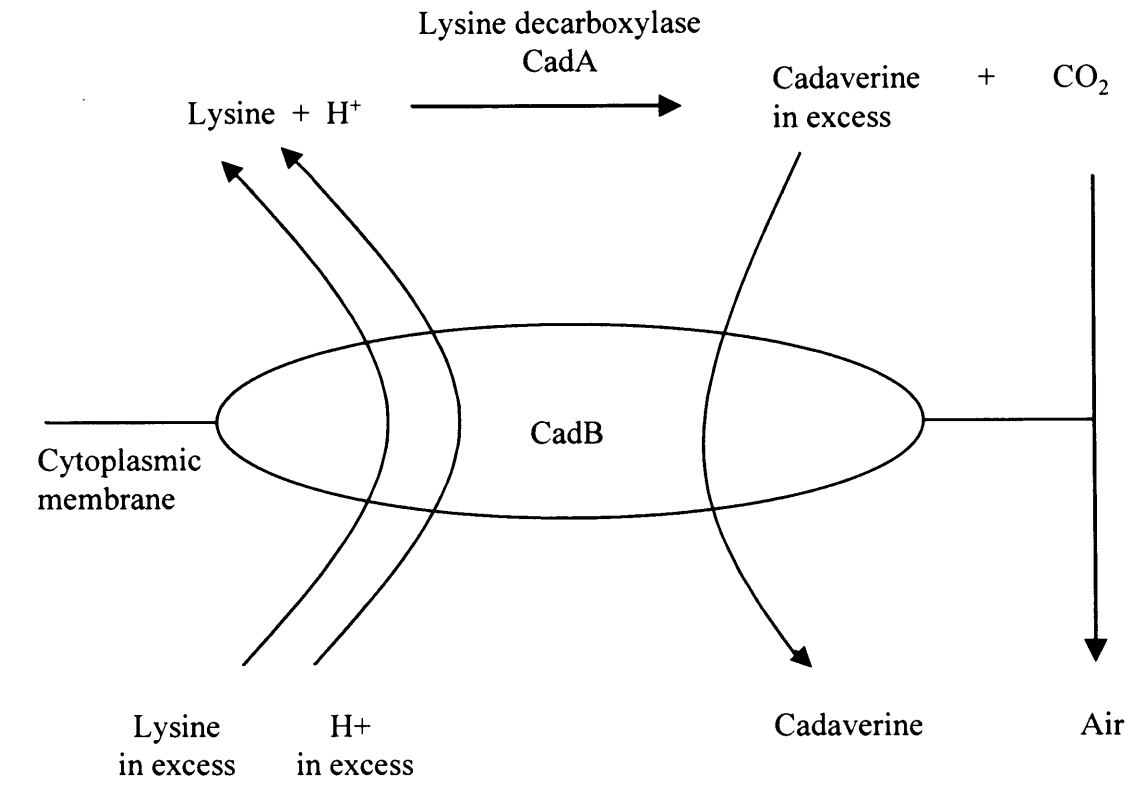

Figure 1.3. A proposal for the mechanism of action of amino acid decarboxylases for the growth of bacteria under acidic conditions and the ability to detoxify external acidic pH.

Lysine is transported into the cell via CadB (lysine/cadaverine antiporter) that then becomes decarboxylated by lysine decarboxylase resulting in the alkaline end-product, cadaverine and carbon dioxide. The external pH is increased by excretion of these end products into the external medium. Protons can also be taken into the cell through the CadB transporter from the external medium and utilised for lysine decarboxylation. Overall, an inward gradient is created that allows lysine and protons to enter the cell and an outward gradient allowing cadaverine and carbon dioxide to leave. After Meng and Bennett (1992a).

has been proposed that cadaverine is excreted from the cell into the external medium via CadB lysine/cadaverine transporter. The lysine/cadaverine transporter in *E. coli* export more cadaverine at pH 5.5 than at pH 8.0 but in strains in which CadB is absent, cadaverine expulsion into the medium is significantly reduced even in the presence of lysine decarboxylase (Meng and Bennett, 1992a). In addition, a high concentration gradient of lysine is required for efficient functioning of CadB (Meng and Bennett, 1992a). A high external proton concentration increased lysine uptake and concurrent expulsion of cadaverine that was not detected in cells grown in minimal medium with low amounts of lysine. This is again supportive of a model for the role of *cadBA* system in regulating pH (Meng and Bennett, 1992a).

A model of CadC activation of P*cad* is based on the finding that the amino-terminal DNA binding domain of CadC is closely related to the carboxy termini of several bacterial proteins resembling bacterial response regulators. The acid-dependent ToxR of *Vibrio cholerae* is one such protein but with the DNA binding domain located at its amino terminus (Watson *et al*, 1992). The model proposed for pH regulation of P*cad* suggests that the amino terminal domain of CadC is the DNA binding domain that exists in the cytoplasm with one or more domains present in the cytoplasmic membrane or periplasmic space. The carboxy terminus senses change in the periplasmic pH and this leads to a conformational change in CadC, in turn signalling to the amino-terminal cytoplasmic domain and subsequently interacting with P*cad* directly or indirectly to promote expression of the *cadBA* operon (Meng and Bennett, 1992a; Watson *et al*, 1992).

The binding site required for activation has been identified on the sequence upstream of P*cad* at –120 base pair using mutational experiments and *in vivo* footprinting in *E. coli* (Meng and Bennett, 1992b; Watson *et al.*, 1992). A 66 base pair fragment containing the regulatory sequence on a high-copy plasmid reduced expression from the chromosomal *cad* operon, signifying that titration of the activator protein was taking place. Single point mutations in this regulatory region led to mutants with altered regions resulting in the loss of protein binding ability and thus, suggesting that part of the fragment was involved in the binding site for CadC (Meng and Bennett, 1992b; Watson *et al.*, 1992).

A hydrophobic membrane protein, LysP (also known as CadR) may also participate in the degradative lysine pathway in *E. coli*. Expression of *lysP* can be induced by the same conditions that induce *cad*BA; anaerobiasis, low pH and lysine (Popkin and Maas, 1980; Tabor *et al.*, 1980; Steffes *et al.*, 1992, Neely *et al.*, 1994). The *lysP* gene has been mapped to approximately minute 46.5 on the *E. coli* chromosome (Tabor *et al.*, 1980; Steffes *et al.*, 1992). Lysine mutants are resistant to thiosine, a lysine analog, however, thiosine is inhibitory to *lysP*, suggesting that LysP is a specific lysine transporter as decreased levels of lysine transport were reported with the addition of thiosine (Steffes *et al.*, 1992). Also, a recombinant *E. coli* with a plasmid carrying *lysP* displayed a 10- to 20- fold increase in the uptake of lysine indicating a role for LysP as a lysine transporter (Steffes *et al.*, 1992). LysP is also a regulator of lysine decarboxylase because mutations of the *lysP* gene gave rise to increased levels of lysine decarboxylase (Tabor *et al.*, 1980). A negative function of LysP has also been indicated (Neely *et al.*, 1994). Overproduction of LysP prevented induction of *cadA* but expression of *cadA* was not affected by LysP if *cadC* was absent. Therefore it appears that LysP functions through CadC (Neely *et al.*, 1994).

The *hns* gene product, H-NS, a histone-like protein, has also been implicated in derepressing *cadA* expression. This has been shown following a mutation in *hns* at neutral external pH 8.0 (Shi and Bennett, 1995). The gene, *leuO*, was found to complement the *hns* mutant phenotype and suppressed the effect of a *hns* mutation on *cadA* expression (Shi and Bennett, 1995). Excess LeuO protein acts by repressing the expression of the activator *cadC* at the transcriptional level, possibly through LeuO binding directly to the *cadC* promoter sequences (Shi and Bennett, 1995).

As stated previously (page 65), it has been assumed that cadaverine excretion into the medium would result in neutralisation of the medium and survival at low pH (Meng and Bennett, 1992a). However other experiments have not substantiated this hypothesis. Large amounts of cadaverine excreted into the medium were found only to increase the external pH slightly suggesting a different role for the polyamine in *E. coli* (Samartzidou and Delcour, 1999). Polyamines have been shown to decrease the permeability of the membrane by closure of porins; these are trimeric proteins that are mostly open channels. Two mechanisms in response to an acidic pH have been put forward. Firstly, cadaverine-dependent inhibition of porin-mediated fluxes has been

observed, thus cadaverine could act as an endogenous modulator of porin-mediated outer membrane permeability. The expression of *cadC* in the regulation of porin expression has also been suggested. Sequences in the promoter region of *ompF* and *ompC* were shown to be similar to the *cadBA* promoter region to which the CadC protein interacts. A reduction in porin level and inhibition of outer membrane porin genes *ompF* and *ompC* expression was seen to occur through the interaction of CadC protein with the porin promoters (Samartzidou and Delcour, 1999).

A further model has been proposed for the *cad* operon, which is in disagreement to that previously suggested (Meng and Bennett, 1992a; Watson *et al.*, 1992). This model implies that the *E. coli cad* operon functions as a supplier of carbon dioxide under oxygen limiting conditions at low pH (Takayama *et al*, 1994). In the previous model, no feasible explanation was given for the expression of the *cad* operon under oxygen limiting conditions (Meng and Bennett, 1992a; Watson *et al.*, 1992). The pH range for the growth of *E. coli* is pH 6.0 to pH 8.0 and the expression of the *cad* operon was maximal at pH 6.3 under oxygen limiting conditions (Takayama *et al*, 1994). Therefore, growth of *E. coli* at pH 6.3 is not detrimental to the cell and consequently lysine decarboxylase is not produced to increase acidic external pH. The model suggests that under anaerobic conditions, the Krebs cycle does not function and subsequently leads to a reduction in carbon dioxide since the Krebs cycle involves the production of energy and carbon dioxide. Under these conditions the *cad* operon is induced in order to supply carbon dioxide to the cell (Takayama *et al*, 1994). Furthermore, high levels of carbonate (or its derivatives) repressed the *cad* operon indicating that carbon dioxide regulates the expression of the operon (Takayama *et al*, 1994).

CHAPTER 2: MATERIAL AND METHODS.

2.1. Chemicals.

Unless otherwise stated all chemicals were obtained from Sigma Chemical Company Ltd (U.K.) or BDH Ltd (U.K.).

2.2. Bacterial Strains and Plasmids.

The bacterial strains and plasmids used in this study are shown in Tables 2.1 and 2.2. *Escherichia coli* strain DH5α was grown in Luria broth or Luria agar at 37°C. *Mycobacterium smegmatis* mc^2155, *Mycobacterium aurum*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* were grown in 7H9 broth supplemented with Albumin-Dextrose-Catalase (ADC) or 7H11 agar supplemented with Oleic acid-Albumin-Dextrose-Catalase at 37°C. *Mycobacterium smegmatis* could also be grown on Luria agar. *Escherichia coli* harbouring *E. coli*-mycobacteria shuttle vectors conferring hygromycin resistance were grown in the presence of 200 μg/ml of hygromycin B. Mycobacteria containing vectors conferring hygromycin resistance were grown in the presence of 50 μg/ml of hygromycin B. Bacteria harbouring vectors conferring ampicillin resistance were grown in the presence of 100 μg/ml of ampicillin. Bacteria harbouring vectors conferring kanamycin resistance were grown in the presence of 25 μg/ml of kanamycin. *E. coli* DH5α harbouring cosmids MTCY159 and MTCY369 were grown in the presence of 50 μg/ml of ampicillin.

2.2.1. Growth Media.

Media were prepared using distilled water and sterilised by autoclaving at 121°C at 15 pounds per square inch (psi) for 15 minutes.

Luria broth:	10 g Tryptone, 5 g Yeast extract, 5 g NaCl, 1000 ml Distilled water.
Luria agar:	1.5% (w/v) Agar (Lab M) was added to Luria broth.
7H9 broth:	1.04 g 7H9 base (Difco), 500 μl 20% (v/v) Tween 80, 200 ml Distilled water. After autoclaving 20 ml of ADC enrichment was added to 180 ml of the cooled medium.

Table 2.1. Bacterial Strains

All strains were obtained from the Leicester University Culture Collection.

Strain	Genotype and/or description	Reference
Escherichia coli		
DH5α	supE44 ΔlacU169 (Φ80 lacZΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan, (1983)
Mycobacteria		
Mycobacterium smegmatis mc^2155	Mutant strain with a high transformation phenotype	Snapper *et al.*, (1990)
Mycobacterium aurum 4721E	Type strain	Institut Pasteur
Mycobacterium bovis BCG	Attenuated strain.	Evans Medical Ltd., U.K.
Mycobacterium tuberculosis H37Rv.	Type strain	NCTC 7416

Table 2.2. Bacterial plasmids

Plasmid	Description	Reference
pOLYG	*E. coli*-mycobacteria shuttle vector conferring hygromycin resistance.	O'Gaora *et al.*, (1997)
pSMT3	pOLYG with a promoter for the gene for the mycobacterial heat shock protein *hsp*60, conferring hygromycin resistance.	O'Gaora *et al.*, (1997)
pSMT1	pSMT3 expressing *Vibrio harveyi luxAB* genes downstream from a *hsp*60 promoter, conferring hygromycin resistance.	Snewin *et al.*, (1999)
pUC19	High copy number *E. coli* vector with blue-white selection conferring ampicillin resistance.	Yanisch-Peron *et al.*, (1985)
pBluescript	pBluescript II KS (+/-) phagemid derived from pUC19, a *lac* promoter for gene expression, blue-white selection, conferring ampicillin resistance.	Stratagene (U.K.)
pPCR-Script	pPCR-Script Amp SK (+) cloning vector is based upon pBluescript SK (+) phagemid with an *Srf* I site incorporated, conferring ampicillin resistance.	Stratagene (U.K.)
pSB226	PCR *luxAB* cloned into pHG177 vector, conferring ampicillin resistance.	Hill *et al.*, (1991)
pJS11	pOLYG carrying *Vibrio harveyi luxAB* genes from pSB226, conferring hygromycin resistance.	This study
MTCY159	*Mycobacterium tuberculosis* H37Rv cosmid (pYUB328) carrying gene homologous to an amino acid decarboxylase, Rv2531c.	Institut Pasteur
MTCY369	*Mycobacterium tuberculosis* H37Rv cosmid (pYUB328) carrying gene homologous to a two-component regulator, *phoPR*.	Institut Pasteur
pJS14	PCR 301 bp upstream region of Rv2531c cloned into pBluescript, conferring ampicillin resistance.	This study
pJS15	PCR 1065 bp upstream region of MTCY369 (*phoPR*) cloned into pBluescript, conferring ampicillin resistance.	This study

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Table 2.2. Bacterial Plasmids Continued:

Plasmid	Description	Reference
pJS6	*luxCDABE* genes cloned downstream of *hsp*60 promoter of pSMT3 with directional transcription, conferring hygromycin resistance.	This study
pMV261	*E. coli-Mycobacterium* shuttle vector, *hsp*60 promoter, conferring kanamycin resistance.	Stover *et al.*, (1991)
pPA3	*Vibrio harveyi luxAB* genes cloned downstream of *hsp*60 promoter of pMV261.	Andrew and Roberts (1993)
pSP1	*Photorhabdus luminescens luxAB* genes cloned downstream of *hsp*60 promoter of pSMT3, conferring hygromycin resistance.	This study
pRDH42	*Photorhabdus luminescens luxAB* genes and TL1 terminator sequence from lambda cloned in to pBluescript, conferring ampicillin resistance.	Purdy and Park (1993) (Gift from Haigh, University of Leicester)

ADC enrichment: 5 g Bovine albumin fraction V (First Link Ltd, U.K.), 2 g D (+) Glucose (Dextrose), 3 mg Beef catalase, 100 ml Distilled water. The medium was filter sterilised through a 0.2 μm Vacucap (Sterilin).

7H11 agar: 10.5 g 7H11 agar (Difco), 2.5 ml Glycerol, 450 ml Distilled water. After autoclaving 50 ml of OADC enrichment was added to 450 ml of the cooled medium.

OADC enrichment: 56.18 μl Oleic acid, 5 g Bovine albumin fraction V, 2 g D (+) Glucose (Dextrose), 0.004 g of Beef catalase, 100 ml Distilled water. The medium was filter sterilised through a 0.2 μm Vacucap (Sterilin).

Dubos broth: 13 g Dubos base (Difco)(Double strength), 2 g Glucose, 1000 ml Distilled water.

2.3. Extraction of DNA.

Plasmid DNA extraction was based on the method described by Sambrook and others (1989) using the following reagents:

Solution I: 50 mM Glucose (filter sterilised), 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0) (Diaminoethanetetra-acetic acid disodium salt (Fisons)).

Solution II: 0.2 mM NaOH, 1% (w/v) SDS.

Solution III: 60 ml 5 M Potassium acetate, 11.5 ml Glacial acetic acid, 28.5 ml Distilled water.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

2.3.1. Small-Scale Extraction of Plasmid DNA.

Luria broth was inoculated with a fresh culture taken from an agar plate and incubated overnight at 37°C with shaking at 200 rpm. The culture was harvested using 3.0 ml, centrifuged at 6,000 rpm for 1 minute using a Micro Centaur bench-top microfuge. The supernatant was discarded and the resulting bacterial pellet was resuspended in 200 µl of Solution I. After incubation at room temperature for 5 minutes, 400 µl of Solution II was added. Inverting the tube 4-6 times mixed the solution gently but thoroughly, this was followed by a 5 minute incubation on ice. 225 µl of 10 M ammonium acetate was added, mixed immediately but gently and incubated on ice for a further 5 minutes. The tube was centrifuged in a Micro Centaur microfuge at 13,000 rpm for 10 minutes, to remove particulate material. 750 µl of supernatant was removed immediately into a fresh tube and 480 µl of isopropanol (propan-2-ol) was added to precipitate the DNA. The tube was incubated at room temperature for 10 minutes. Plasmid DNA was collected by centrifugation at 13,000 rpm for 10 minutes in a Micro Centaur microfuge. The DNA pellet was washed in 70% (v/v) ethanol, pulsed-centrifuged to remove residual ethanol and dried at room temperature. The DNA pellet was resuspended in 50 µl of TE buffer.

2.3.2. Large-Scale Extraction of Plasmid DNA.

From a fresh agar plate, 5 ml of Luria broth was inoculated with culture and incubated overnight at 37°C with shaking at 200 rpm. The overnight culture was inoculated into 500 ml of fresh Luria broth and incubated overnight at 37°C with shaking at 200 rpm. Bacterial cells were harvested by centrifugation at 7,000 rpm for 10 minutes in a Sorvall GSA centrifuge. The supernatant was removed and the resulting pellet was resuspended in 25 ml of Solution I containing 10 mg of lysozyme. After 10 minutes incubation at room temperature, 50 ml of Solution II was added. The solution was mixed gently but rapidly by inversion to produce a clear lysate. 37.5 ml of cold Solution III was added, mixed thoroughly by gentle inversion and incubated on ice for a following 10 minutes. Particulate material was removed by centrifugation at 10,000 rpm for 30 minutes at 4°C in a Sorvall GSA centrifuge. The supernatant was filtered through a 5 ml Gilson tip plugged with polymer wool into 0.6-volume isopropanol (propan-2-ol) and incubated at room temperature for 10 minutes. The precipated DNA was collected by centrifugation at 10,000 rpm in a Sorvall GSA centrifuge at room temperature to prevent salt co-

precipitating. The supernatant was carefully poured off and the pellet was washed in approximately 5 ml of 70% (v/v) ethanol, dried and dissolved in 3 ml of TE buffer.

3 ml of cold 5 M $LiCl_2$ was added and mixed to precipitate the high-molecular weight RNA. The solution was centrifuged at 10,000 rpm in a Sorvall SS-34 centrifuge for 10 minutes to pellet the RNA. The supernatant was added to an equal volume of isopropanol (propan-2-ol) and mixed by inversion. The DNA was pelleted at 10,000 rpm in a Sorvall SS-34 centrifuge for 10 minutes, washed with 70% (v/v) ethanol and dried. The DNA was dissolved in 500 µl of TE, DNAse-free Rnase solution at 20 µg/ml and incubated at 37°C for 30 minutes. The optional step consisted of PEG precipitation. To the solution, 500 µl of 1.6 M NaCl containing 13% (w/v) PEG was added and incubated on ice for 10 minutes. The solution was centrifuged in a Micro Centaur bench-top microfuge for 10 minutes at 13,000 rpm. The supernatant was carefully removed and the DNA pellet dissolved in 400 µl of TE buffer, followed by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1). Extractions were continued until no white material could be seen. The phases were separated by centrifugation at 13,000 rpm for 2 minutes in a Micro Centaur microfuge. Removal of residual phenol required the extraction of the solution with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated by addition of 100 µl of 10 M ammonium acetate and 1 ml of absolute ethanol. After incubation at –20°C for 30 minutes the DNA was recovered by centrifugation in a Micro Centaur microfuge for 10 minutes at 13,000 rpm. The DNA was washed in 70% (v/v) ethanol, dried and dissolved in 100-200 µl of TE buffer.

2.3.3. Extraction of Mycobacterial Plasmid DNA.

Plasmid DNA was extracted from *Mycobacterium smegmatis* using a modification of the method of Kado and Liu (1981) using the following reagents:

E Buffer: 40 mM Tris-acetate (pH 7.9), 2 mM EDTA.

Lysis Solution: 3% (w/v) SDS, 0.6 M NaOH.

Mycobacterium smegmatis mc^2155 harbouring recombinant shuttle-plasmid was grown in 7H9 broth containing the appropriate antibiotic at 37°C for 48 hours with shaking at 200 rpm. 5 ml of this culture was inoculated into 5 ml of fresh 7H9 broth containing the appropriate antibiotic and 2 mg/ml D-cycloserine. The culture was incubated for a further 18-24 hours at 37°C with shaking at 200 rpm. Cells were collected by centrifugation at 5000 rpm for 10 minutes in a Heraeus Christ centrifuge, washed in 1 ml PBS (Phosphate Buffered Saline pH 7.2) and centrifuged in a Micro Centaur bench-top microfuge at 6,000 rpm for 3 minutes. The cell pellet was resuspended in 100 µl of E Buffer and incubated for 10 minutes at room temperature. Followed by the addition of 200 µl of fresh Lysis Solution and incubation for 30 minutes at 56°C. After two extractions with phenol:chloroform:isoamyl alcohol (24:24:1) and two chloroform:isoamyl alcohol (24:1) extractions, the DNA was precipitated from the final aqueous phase with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol, the tube was incubated at –20°C for 30 minutes. The DNA was recovered by centrifugation at 13,000 rpm for 10 minutes in a Micro Centaur microfuge and the resulting pellet was washed in 70% (v/v) ethanol, dried and dissolved in 25 µl of nanopure water. 5 µl of the DNA preparation was used to transform *Escherichia coli* DH5α (section 2.8.1.2.), as the final DNA yield from the preparation could not be satisfactorily visualised by agarose gel electrophoresis. Extraction of plasmid DNA from *E. coli* transformants gave greater yield and quality of DNA.

2.3.4. Extraction of Mycobacterial Chromosomal DNA.

Extraction of mycobacterial chromosomal DNA was based on the method of Davis and others (1991) with modifications using the following reagents:

Wash Buffer:	0.3 M Sucrose (filter sterilise), 50 mM Tris-HCl (pH 8.0), 10 mM EDTA.
GSE Solution:	6 M Guanidium hydrochloride, 1% (v/v) Sarkosyl, 10 mM EDTA.

For fast growing mycobacteria (such as *Mycobacterium smegmatis* mc^2155), 20 ml of a 2-day grown culture was inoculated into 180 ml of double strength Dubos broth base or

7H9 broth. Incubation was continued for a further 2 days at 37°C with shaking at 200 rpm. For slow growing mycobacteria (such as *Mycobacterium bovis* BCG), 20 ml of a 7 day grown culture was inoculated into 180 ml of medium and incubation continued for a following 1-2 weeks at 37°C stationary. Cultures were harvested at 4000 rpm for 15 minutes at 4°C in a Heraeus Supertech Minifuge T centrifuge and the cell pellet washed in Wash Buffer. Cells were resuspended at about 2ml/g wet-weight in the wash buffer containing 2 mg/ml lipase and 2 mg/ml lysozyme followed by incubation at 37°C for 60 minutes. Four times the volume of GSE Solution was added and incubation continued for a further 2 hours at 37°C. The solution was extracted once with an equal volume of chloroform. DNA was precipitated overnight at –20°C using 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol.

The precipitated DNA was centrifuged at 4000 rpm for 15 minutes in a Heraeus Supertech Minifuge-T centrifuge, washed in 70% (v/v) ethanol, dried and resuspended in 400-800 µl of TE buffer containing Proteinase K (0.5 mg/ml) and Rnase T1 (4,000 Units) and incubated for 3 hours at 37°C. The chromosomal DNA was extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform:isoamyl alcohol (24:1). The phases were separated by centrifugation at 13,000 rpm for 2 minutes in a Micro Centaur microfuge. Addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol precipitated the chromosomal DNA. The tube was centrifuged at 13,000 rpm in a Micro Centaur microfuge and the DNA pellet washed in 70% (v/v) ethanol, dried and dissolved in 100 µl of TE buffer. The mycobacterial chromosomal DNA was stored at 4°C.

2.3.5. Extraction of Total DNA from *Mycobacterium bovis* BCG.

Extraction of total DNA from *Mycobacterium bovis* BCG was based on the method of Ruth McAdam (personal communication) using the following reagents:

GTE Solution 9.5:	50 mM Glucose (filter sterilised), 100 mM Tris-HCl (pH 9.5), 20 mM EDTA (pH 8.0).
Lysozyme:	Fresh 100 mg/ml in GTE.

CTAB: 4.1g NaCl was added into 80 ml distilled water, while stirring, 10g CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide) was added, heated to 65°C and made up to a volume of 100 ml.

TO.1E Buffer: 10 mM Tris-HCl (pH8.0), 0.1 mM EDTA (pH 8.0).

Mycobacterium bovis BCG was grown in 10 ml of 7H9 broth at stationary to late log phase with antibiotic selection at 37°C. The culture was harvested at 3,000 rpm for 20 minutes in a Heraeus Christ centrifuge, the supernatant poured off and the pellet frozen at –20°C. The cell pellet was thawed at room temperature, resuspended in 0.5 ml of GTE solution and carefully transferred to a microfuge tube and heat killed at 80°C for 30 minutes. The suspension was cooled and 50 µl of lysozyme was added and incubated at 37°C overnight. 70 µl of SDS was added, mixed, followed by 6 µl of proteinase K at 20 mg/ml and incubated at 56°C for 30 minutes. To the suspension, 100 µl of prewarmed NaCl and 80 µl of prewarmed CTAB were added, mixed, followed by incubation at 56°C for 15 minutes. The addition of CTAB turned the suspension white. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed by sharp shakes and then centrifuged at 13,000 rpm for 5 minutes in a Micro Centaur bench-top microfuge. To precipitate the DNA the upper aqueous phase was transferred to a microfuge tube containing 0.6-volumes of isopropanol, mixed by inverting and centrifuged at 13,000 rpm for 15 minutes in a Micro Centaur microfuge. The pellet was washed once in 1 ml of 70% (v/v) ethanol, spun to remove residual ethanol and dried at room temperature for 10 minutes. The pellet was dissolved overnight at 4°C in 20 µl of T0.1E buffer. The total DNA was used to transform *E. coli* DH5α (section 2.8.1.2).

2.4. Purification of DNA from Agarose Gels.

Plasmid DNA was extracted from agarose gels using the Sephaglas Bandprep Kit from Pharmacia Biotech with some modifications. The components of the Sephaglas Bandprep Kit included the following:

Sephaglas BP:	20% (w/v) Sephaglas BP suspended in 6 M NaI, 50 mM Tris-HCl (pH 8.0), 0.05% (w/v) Na_2SO_4 and 10 mM CDTA (trans-1,2-Diaminocyclohexanetetra-acetic acid).
Gel Solubilizer:	6 M NaI, 50 mM Tris-HCl (pH 8.0), 0.05% Na_2SO_4 and 10 mM CDTA.
Elution Buffer:	10 mM Tris-HCl (pH 8.0), 1 mM EDTA.
Wash Buffer:	20 mM Tris-HCl (pH 8.0), 1 mM CDTA and 0.1 mM NaCl solution to which 18 ml of absolute ethanol was added before use.

Using a clean scalpel, the slice of agarose containing the DNA band to be extracted was excised, cutting as close as possible to the band. The slice was cut into several smaller pieces; 500 µl of Gel Solubilizer was added, vortexed vigorously and incubated at 60°C for 5-10 minutes or until the agarose slice had dissolved. The container of Sephaglas BP was shaken to form a uniform suspension; 10 µl of suspension was added to the dissolved gel slice and vortexed gently. The tube was incubated at room temperature for 5 minutes, vortexing gently every minute to resuspend the sephaglas, followed by pulse spin for 10 seconds in a Micro Centaur bench-top microfuge. The supernatant was carefully discarded, taking care not to disturb the Sephaglas pellet. 80 µl of Wash Buffer was added to the Sephaglas pellet and pipetted up and down several times to resuspend the pellet. After a pulse spin for 10 seconds in a Micro Centaur microfuge, the supernatant was carefully removed and this step was repeated for a total of three washes. Following the last wash and removal of supernatant, the tube was vortexed gently to partially disperse the Sephaglas pellet and allowed to air-dry at room temperature for 10 minutes. DNA was eluted by adding 20 µl of Elution Buffer to the tube, vortexed gently to resuspend the Sephaglas pellet and incubated at room temperature for 5 minutes with periodic agitation. Subsequently, the DNA was recovered by centrifugation at 13,000 rpm for 1 minute in a Micro Centaur microfuge. The supernatant was removed and placed in a clean tube, taking care not to disturb the Sephaglas pellet. The elution step was repeated once more to obtain a better yield of DNA.

2.5. PCR Reactions.

PCR reactions (Saiki *et al.*, 1988) were prepared as follows using the following reagents:

1X ThermoPol Reaction Buffer:	10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8, @ 25°C), 2 mM MgSO4, 0.1% Triton X-100.

The PCR was done in 50 µl or 100 µl volumes. The reaction mix contained 1X ThermoPol reaction buffer (New England BioLabs, U.K.), 200 µM of each of the following deoxynucleotides: dATP, dTTP, dGTP and dCTP (Advanced Biotechnologies Ltd), forward and reverse primer up to 100 pmoles (Table 2.3.), magnesium sulphate ($MgSO_4$) at a final concentration of 2 to 6 mM, template DNA up to 100 ng and sterile nanopure water made up to the final volume. Reactions were overlaid with 100 µl of mineral oil (molecular biology grade, Sigma: oil was exposed to UV light for 15 minutes to destroy any DNA contaminants), placed into the Hybaid OmniGene thermal cycler and heated at 94°C for 5 minutes for 1 cycle (hot start). 0.5 µl of $Vent_R^{®}$ (exo-) DNA polymerase (New England BioLabs, U.K.) was added to each tube and amplification continued for approximately 30 to 35 cycles. Thermal cycle parameters were as follows:

Denaturation:	95°C for 1 minute
Annealing:	52°C for 1 minute
Extension:	72°C for 2 minutes

These cycles were followed by 1 cycle at 72°C for 10 minutes and holding the temperature at 4°C until the PCR tubes were removed and stored at –20°C. The PCR products were analysed by using electrophoresis through a 0.7% (w/v) agarose gel as described (section 2.11.1).

Table 2.2. Bacterial Plasmids Continued:

Plasmid	Description	Reference
pJS12	301 bp upstream region of Rv2531c from pJS14 cloned into pJS11, conferring hygromycin resistance.	This study
pJS13	1065 bp upstream region of MTCY369 (*phoPR*) from pJS15 cloned into pJS11, conferring hygromycin resistance.	This study
pJS16	1065 bp upstream region of MTCY369 (*phoPR*) from pJS15 cloned into pJS11, transcribing in the opposite direction to *luxAB* conferring hygromycin resistance.	This study
pSM20	Suicide vector carrying *sacRB* cassette mediating sucrose sensitivity, conferring ampicillin resistance.	Selbitschka *et al.*, (1993)
pUC-4K	Kanamycin cassette conferring resistance to kanamycin from the transposon Tn 903.	Pharmacia
pJS20	PCR Rv2531c complete gene from MTCY159 cloned into pPCR-Script, ampicillin resistance marker.	This study
pJS21	Rv2531c from pJS20 cloned into pSM20, ampicillin resistance marker.	This study
pJS22	Kanamycin cassette from pUC-4K cloned into pJS21, conferring resistance to ampicillin and kanamycin..	This study
pJS23	Hygromycin resistance marker from pSMT3 cloned into pJS22, conferring, resistance to ampicillin, kanamycin and hygromycin.	This study.
pJS23-dc	Rv2531c knockout in *M. smegmatis* carrying pJS23, double-crossover conferring hygromycin resistance.	This study
pSfi390	*lux* operon (*CDABE*) from *Photorhabdus luminescens*, strain Hb (ATCC 29999), conferring ampicillin resistance.	Swift *et al.*, (1995)
pJS5	*luxCDABE* genes cloned downstream of *hsp*60 promoter of pSMT3 with apposing transcription, conferring hygromycin resistance.	This study

Table 2.1. Bacterial Strains

All strains were obtained from the Leicester University Culture Collection.

Strain	Genotype and/or description	Reference
Escherichia coli		
DH5α	supE44 ΔlacU169 (Φ80 lacZΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan, (1983)
Mycobacteria		
Mycobacterium smegmatis mc^2155	Mutant strain with a high transformation phenotype	Snapper *et al.*, (1990)
Mycobacterium aurum 4721E	Type strain	Institut Pasteur
Mycobacterium bovis BCG	Attenuated strain.	Evans Medical Ltd., U.K.
Mycobacterium tuberculosis H37Rv.	Type strain	NCTC 7416

Table 2.3. PCR Primers and Reaction Parameters.

Primer name/Sequence/Size

LuxA-HB (for)	5'ATGAAATTTGGAAACTTTTTGCTTACA3'	27 mer
LuxB-HB (rev)	5'TTAGGTATATTCCATGTGGTACTT3'	23 mer

Annealing temperature = 1 minute at 52°C

Extension temperature = 2 minutes at 72°C

LuxC-HB1 (for)	5'TCATTCATTATTAACGGCCAGGTT3'	23 mer
LuxC-HB2 (rev)	5'TTATGGGACAAATACAAGGAAGTT3'	24 mer

Annealing temperature = 1 minute at 52°C

Extension temperature = 2 minutes at 72°C

LuxD-HB1 (for)	5'TCAAAATATAAAACCATCGACCACGTT3'	27 mer
LuxD-HB2 (rev)	5'AGACAGAGAAATTGCTTGATTTTCAAT3'	27 mer

Annealing temperature = 1 minute at 52°C

Extension temperature = 2 minutes at 72°C

MTCY159-F (for)	5'GC**ATCGAT**CACGCCGCATCAGCACTG3'	26 mer
MTCY159-R (rev)	5'CCC**AAGCTT**GTCGTCACCTGTTCTCTGG3'	28 mer

Annealing temperature = 1 minute at 52°C

Extension temperature = 2 minutes at 72°C

MTCY369-F (for)

5'CCC**ATCGAT**GC**AAGCTT**GATCAGCCGAACCAACTGTC3' 37 mer

MTCY369-R (rev)

5'CCC**ATCGAT**TGGTTGAACGTTACCTTCACA3' 30 mer

Annealing temperature = 1 minute at 45°C

Extension temperature = 2 minutes at 72°C

Table 2.3. PCR Primers and Reaction Parameter Continued.

Rv2531c -FOR	5'ATGAACCCAAACAGCGTCCGC3'	21 mer
Rv2531c -REV	5'TCACGCGTCTTCGGCGAC3'	18 mer
Rv2531c -FOR1 (for)	5'GC**AAGCTT**TCGATGACGCGTGTCGTCAC3'	28 mer
Rv2531c -REV2 (rev)	5'GC**AAGCTT**GCCATTCAAAGCCGAAGCG3'	27 mer

Annealing temperature = 1 minute at 52°C

Extension temperature = 6 minutes at 72°C

HygF	5'CCTACCTGGTGATGAGCCGGA3'	21 mer
HygR	5'GGTCTCCTCGAACACCTCGAA3'	21 mer

Annealing temperature = 30 seconds at 52°C

Extension temperation = 30 seconds at 72°C

Note - **ATCGAT** represents a *Cla* I restriction site.

AAGCTT represents a *Hind* III restriction site.

2.6. Automated DNA Sequencing.

The sequencing method used is a modification of the chain termination method developed by Sanger and others (1977). Automated DNA sequencing used the ABI PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems) in combination with an Applied Biosystems Model 377 or 377XL DNA sequencing system. The cycle sequencing kit contains several components, including a mixture of deoxy- and dideoxynucleotides that are used in the reaction. Incorporation of dye-labelled dideoxynucleotides blocks further chain elongation to a produce a dye labelled reaction product. Also included were deoxynucleotides contained dITP to replace dGTP to minimise band compressions and AmpliTaq DNA Polymerase FS (Applied Biosystems) a modified DNA polymerase to resemble that of T7 DNA polymerase. This polymerase effectively has no 5' to 3' nuclease activity and has a modified active site that accommodates dideoxynucleotides more easily than the unmodified form of the enzyme, leading to more uniform band intensities (Parker *et al.*, 1996). The products of the reaction are analysed colourimetrically on the 377 or 377XL sequencing workstation.

The sequencing reactions were done using 400 ng of double stranded plasmid DNA that was mixed with 3.2 pmole of primer and 8 µl of Terminator Ready Reaction Mix and made up to a final volume of 20 µl (Terminator Mix contained the 4 dye-labelled dideoxynucleotides, dATP, dCTP, dTTP, dITP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphate and AmpliTaq DNA Polymerase) (Qiagen, 1998). Reactions were done in duplicate. The reactions were overlaid with 40 µl of UV irradiated mineral oil, placed in a Perkin-Elmer GeneAmp™ PCR System 9600 and 9700 thermal cycler and thermal cycling was done as follows:

Denaturation:	Rapid thermal ramp to 95°C Held at 95°C for 30 seconds
Annealing:	Rapid thermal ramp to 50°C Held at 50°C for 10 seconds
Extension:	Rapid thermal ramp to 60°C Held at 60°C for 4 minutes

Repeated for 25 cycles.

After completion of thermal cycling, any unincorporated terminators were removed by ethanol precipitation. To a tube containing 2 µl of 3 M sodium acetate and 50µl of 95% (v/v) ethanol, the 20 µl reaction mixture was added. After vigorous mixing the sample was placed on ice for 10 minutes followed by centrifugation at 13,000 rpm in a Micro Centaur bench-top microfuge for 20 minutes. The supernatant was removed and the resulting pellet washed in 250 µl of 70% (v/v) ethanol. The ethanol was carefully removed and the pellet air-dried. The reaction products were analysed on an ABI Model 373 or 377XL DNA sequencer by Dr. Katherine Lilly at the Protein and Nucleic Acid Sequencing Laboratory, University of Leicester (http://www.le.ac.uk/cmht/pnacl.html). Sequence data were analysed using the SeqEd program supplied by Applied Biosystems Inc, and the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

2.7. Cloning of PCR Fragments into PCR-Script™.

The PCR-Script™ Amp SK (+) cloning kit from Stratagene was used to clone in fragments of PCR DNA as described in the supplier's protocol:

SOC Medium: 20 g Tryptone, 5 g Yeast extract, 0.5 g NaCl, 1000 ml distilled water. After autoclaving 5 ml of 2 M $MgCl_2$ and 5 ml of 2 M $MgSO_4$ was added. 20 ml of 1 M Glucose (filter sterilised) was added before use.

2.7.1. Ligating the PCR Products into the PCR-Script Amp SK (+) Cloning Vector.

To prepare the cloning reaction, the following components were added in order into a 0.5 ml microfuge tube: 1 µl of the pPCR-Script Amp SK (+) cloning vector at 10 ng/µl, 1 µl of PCR-Script 10X reaction buffer, 0.5 µl of 10 mM rATP, 2-4 µl of the PCR product, 1 µl of *Srf* I restriction enzyme at 5 U/µl, 1 µl of T4 DNA ligase at 4 U/µl and distilled water to a final volume of 10 µl. The cloning reaction was mixed gently and incubated for 1 hour at room temperature and then heated for 10 minutes at 65°C. The cloning reaction was stored on ice until ready to use for transformation into the Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells.

2.7.2. Transformation of Epicurian Coli® with Ligation Mix.

The Epicurian Coli® XL1-Blue MRF′ Kan supercompetent cells (Stratagene) were thawed on ice. The cells were mixed gently and 40 µl of the supercompetent cells were aliquoted into pre-chilled 15 ml Falcon 2059 polypropylene tubes. To prepare the transformation reaction, 0.7 µl of β-mercaptoethanol (1.44 M) was added to the polypropylene tubes containing the supercompetent cells to yield a final concentration of 25 mM. The transformation reaction was swirled gently and incubated on ice for 10 minutes, swirling the reaction gently every 2 minutes. 2 µl of the cloning reaction (section 2.7.1.) was added to the transformation reaction and swirled gently. The transformation reaction was incubated on ice for 30 minutes. At this stage, the SOC medium was prepared and equilibrated to 42°C. The transformation reaction was incubated in a 42°C water bath for 45 seconds to allow heat pulse. The duration of the heat pulse was critical for optimal transformation efficiencies. The transformation reaction was incubated on ice for 2 minutes. 0.45 ml of the prepared SOC medium was added to the transformation reaction and incubated at 37°C for 1 hour with shaking at 200 rpm. The transformation reaction was plated onto Luria agar containing 100 µg/ml ampicillin, 0.004% (v/v) X-gal in dimethylformamide and 0.1 mM IPTG. The agar plates were incubated at 37°C overnight and white colonies were selected for examination.

2.8. Transformation of Bacterial Cells.

2.8.1. Transformation of *Escherichia coli* using Electroporation.

2.8.1.1. Preparation of Electrocompetent *E. coli.*

Preparation of electrocompetent *E. coli* was based on the method of Dower and others (1988). The purity of the starter culture was checked before commencing by plating out *E. coli* on Luria agar and incubating overnight at 37°C. A fresh overnight culture of 10 ml was inoculated into 1 litre of Luria broth and bacteria were grown shaking at 200 rpm until an OD_{600nm} of 0.6-0.75 was reached (mid-log phase). The culture was chilled on ice for 30 minutes and centrifuged in a chilled rotor (4°C) at 7,000 rpm for 15 minutes in a Sorvall GSA centrifuge. The supernatant was discarded and the pellet washed several times in 20 ml ice-cold sterile nanopure water and centrifuged at 5,000

rpm for 15 minutes at 4°C in a Heraeus Christ centrifuge. The cell pellet was washed several times in ice-cold sterile 10% (v/v) glycerol and centrifuged at 5,000 rpm for 15 minutes at 4°C in a Heraeus Christ centrifuge. The final pellet was resuspended in 1 ml of ice-cold sterile 10% (v/v) glycerol and aliquots were stored at –70°C. The prepared electrocompetent *E. coli* were plated out on Luria agar to check purity.

2.8.1.2. Electrotransformation of *E. coli* with plasmid DNA.

If possible freshly prepared electrocompetent cells were used for transformation or else aliquots previously prepared were thawed on ice and used immediately. 40 µl of cell suspension was placed in a cold 0.2 cm electroporation cuvette (Bio-Rad), 100 ng (volume no greater than 5 µl) of plasmid DNA was mixed with the cell suspension and placed on ice for 1 minute. The Bio-Rad Gene Pulser apparatus was set to 25 µF and 2.5 kV. The Bio-Rad Pulse Controller was set to 200 Ω (Dower *et al.*, 1998). The cold electroporation cuvette was placed in the safety chamber slide and pushed in until the cuvette seated in between the contacts at the base of the chamber. The cuvette was given a single pulse and immediately 1 ml of cold SOC medium was added to the cell suspension, mixed gently, transferred to a sterile tube and incubated for 60 minutes at 37°C stationary. The transformation reaction was plated onto selective media and incubated overnight at 37°C.

2.9. Transformation of Mycobacteria using Electroporation.

2.9.1. Preparation of Electrocompetent *Mycobacterium smegmatis* mc^2155.

Preparation of electrocompetent *M. smegmatis* mc^2155 was based on the method of Snapper and others (1988). A 2-day grown culture of 10 ml *M. smegmatis* was inoculated into 100 ml of fresh 7H9 broth. The culture was incubated at 37°C with shaking at 200 rpm and grown for 14 to 20 hours, until an OD_{600nm} of approximately 1. The culture was incubated on ice for 60 minutes and centrifuged at 7,000 rpm for 15 minutes at 4°C in a Sorvall GSA centrifuge. The cell pellet was washed twice in 100 ml of ice-cold 10% (v/v) glycerol, centrifuged at 7,000 rpm for 20 minutes at 4°C in a Sorvall GSA centrifuge. The resulting pellet was resuspended in 1.2 ml of ice-cold 10% (v/v) glycerol and divided into three aliquots of 400 µl. The aliquots were kept on ice and used immediately.

2.9.2. Electrotransformation of *M. smegmatis* mc^{2}155 with Plasmid DNA.

An aliquot of 400 µl of electrocompetent *M. smegmatis* was placed into a cold 0.2 cm electroporation cuvette (Bio-Rad). The cell suspension was mixed with 1-5 µg of plasmid DNA in a volume no greater than 5 µl. The Bio-Rad Gene Pulser apparatus was set to 25 µF and 2.5 kV. The Bio-Rad Pulse Controller was set to 1000 Ω or affinity. The cold electroporation cuvette was placed in the safety chamber and given a single pulse, immediately 4.5 ml of 7H9 broth was added, mixed gently, transferred to a sterile tube and incubated for 2 hours at 37°C stationary to allow recovery. The transformation reaction was plated onto selective media and incubated at 37°C for 2 days.

2.9.3. Preparation of Electrocompetent *Mycobacterium bovis* BCG.

Preparation of electrocompetent BCG was based on the method of Garbe and others (1994). BCG was inoculated at 1:100 in fresh double strength Dubos broth and grown for approximately 3 weeks at 37°C stationary. 200 ml culture was incubated on ice for 2 hours and centrifuged at 7,000 rpm for 15 minutes at 4°C in a Sorvall GSA centrifuge. The cell pellet was washed twice in 50 ml of ice-cold 10% (v/v) glycerol and centrifuged at 4,000 rpm for 10 minutes at 4°C in a Heraeus Supatech Minifuge T centrifuge. The resulting pellet was resuspended in 1 ml of ice-cold 10% (v/v) glycerol and divided into aliquots of 200 µl. The aliquots were stored at –70°C until required.

2.9.4. Electrotransformation of BCG with Plasmid DNA.

Electrotransformation of BCG with plasmid DNA was based on the method of Dellagostin and others (1993). An aliquot of 200 µl of electrocompetent BCG was placed into a cold 0.2 cm electroporation cuvette (Bio-Rad). The cell suspension was mixed with plasmid DNA up to 2 µg with a volume no greater than 5 µl and placed on ice for 1 minute. The Bio-Rad Gene Pulser apparatus was set to 25 µF and 2.5 kV and the Bio-Rad Pulse Controller was set to 600 Ω. The cold electroporation cuvette was placed in the safety chamber and given a single pulse, immediately 0.9 ml of 7H9 broth was added, mixed gently, transferred to a sterile tube and incubated for 2 hours at 37°C stationary to allow recovery. The transformation reaction was plated onto selective media, wrapped in Nescofilm, placed in Petri-dish bags and incubated at 37°C for approximately 3 to 4 weeks or until colonies were seen.

2.9.5. Preparation of Electrocompetent *Mycobacterium tuberculosis* H37Rv.

Preparation of electrocompetent *M. tuberculosis* H37Rv was based on modified methods of Wards and others (1996) and Zhang and others (1993). *M. tuberculosis* was cultured in 7H9 broth at 37°C stationary until growth was confluent. 500 µl of *M. tuberculosis* culture was inoculated into 10 ml 7H9 broth and incubated for 11 days at 37°C stationary. Cells were harvested by centrifugation at 2,000 rpm for 10 minutes at room temperature in a Mistral 3000i centrifuge. Cells were washed twice in 10% (v/v) glycerol and the resulting pellet resuspended in 100 µl of 10% (v/v) glycerol. The electrocompetent cells of *M. tuberculosis* were used immediately. Cells remained at room temperature at all times.

2.9.6. Electrotransformation of *M. tuberculosis* with Plasmid DNA.

An aliquot of 100 µl of electrocompetent *M. tuberculosis* was placed into a cold 0.2 cm electroporation cuvette (Bio-Rad). The cell suspension was mixed with plasmid DNA up to 2 µg with a volume no greater than 5 µl. The Bio-Rad Gene Pulser apparatus was set to 25 µF and 2.5 kV and the Bio-Rad Pulse Controller was set to 1000 Ω. The cold electroporation cuvette was placed in the safety chamber and given a single pulse, immediately 1 ml of room temperature 7H9 broth was added, mixed gently, transferred to a sterile tube and incubated overnight at 37°C stationary to allow recovery. The transformation reaction was plated onto 7H11 agar containing antibiotic for selection and 50 µg/ml of cycloheximide to prevent growth of fungi. The plates were wrapped in Nescofilm, placed in Petri-dish bags and incubated at 37°C for approximately 3 to 4 weeks or until colonies were seen.

2.10. Detection of Light using X-ray film.

Transformants containing the *lux*AB genes were identified by adding approximately 20 µl of neat decanal onto the lid of a Petri-dish and exposing Cronex (Dupont) film to the transformants in complete darkness for several minutes or longer depending on the intensity of the light signal. Films were processed in an Agfa-Geveart automatic film processor and blackening of the X-ray film identified colonies that produced light. Dark colonies did not expose the X-ray film.

2.11. General DNA Manipulation Techniques.

2.11.1. Agarose Gel Electrophoresis of DNA.

Agarose gel electrophoresis was used to separate DNA fragments using the following reagents:

TAE Buffer:

Stock solution (50X): 242 g Tris-base, 57.1 ml Glacial acetic acid, 0.5 M EDTA (pH 8.0), 1000 ml Distilled water.

Working solution (1X): 100 ml 50X stock, 5 litres of distilled water containing 0.5 µg/ml of ethidium bromide.

The gel was prepared using a concentration of 0.7% (w/v) Agarose (Seakem) to separate DNA fragments greater than 3 Kb, separation of smaller DNA fragments utilised 1% (w/v) Agarose. Agarose was dissolved in TAE buffer pH 7.7 by heating for several minutes, 0.5 µg/ml of ethidium bromide was added to the cooled agarose. Electrophoresis was performed in TAE buffer containing 0.5 µg/ml of ethidium bromide. Loading buffer was added to the DNA samples and the samples then loaded into the wells of the gel. After electrophoresis at constant voltage (less than 20 volts per cm between the electrodes) the DNA fragments were visualised on a long-wave UV transilluminator. The size of the DNA fragments was identified using a 1 Kb DNA ladder (Gibco-BRL).

2.11.2. DNA Restriction Digests.

Restriction endonucleases were obtained from Gibco-BRL and Promega. DNA restriction using restriction endonucleases was done according to the manufacturers' recommendations. Digests were generally done using 0.5 µl (0.5-1 Units) of enzyme, 2 µl of 10X react buffer supplied by the manufacturer, 500 ng of DNA and sterile nanopure water made up to a final volume of 20 µl. Incubation was carried out at 37°C unless otherwise stated. DNA digests using a greater concentration of DNA were carried out in a volume of 100 µl.

2.11.3. Inactivation of Endonucleases.

Inactivation of DNA after DNA restriction endonuclease was performed using phenol:chloroform:isoamyl alcohol (24:24:1) extraction. The DNA was ethanol precipitated and resuspended in sterile nanopure water as described (sections 2.11.6. and 2.11.7).

2.11.4. DNA Dephosphorylation.

The DNA 5'-End Labelling System from Promega was used to dephosphorylate plasmid DNA. Plasmid DNA was digested completely with restriction endonucleases to obtain linear DNA. In the standard reaction, 0.1 units of calf alkaline phosphatase was added to the substrate DNA (up to a total of 10 pmoles of 5' ends), with the addition of 5 µl of 10X calf alkaline phosphatase buffer. The final volume was made up to 50 µl using sterile nanopure water.

For dephosphorylation of protruding 5'-termini, DNA was incubated at 37°C for 30 minutes. A further 0.1 units of alkaline phosphatase was added and incubated for another 30 minutes at 37°C.

For dephosphorylation of recessed 5'-termini or blunt end, DNA was incubated at 37°C for 15 minutes, then at 56°C for 15 minutes. A further 0.1 units of alkaline phosphatase was added and incubation was repeated at both temperatures.

The reaction was stopped using phenol:chloroform:isoamyl alcohol (24:24:1) extraction. The DNA was ethanol precipitated and resuspended in sterile nanopure water or TE buffer (sections 2.11.6. and 2.11.7).

2.11.5. DNA Ligation.

T4 DNA ligase (Gibco-BRL) was used to join DNA fragments with staggered or blunt ends. To obtain the optimal ratio of vector to insert DNA for ligation, a 1:3 molar ratio of vector:insert was recommended. Ligations were performed using 2 units of T4 DNA ligase, dephosphorylated vector DNA, insert DNA, 4 µl of Ligase Reaction Buffer supplied by the manufacturer and sterile nanopure water to a final reaction volume of 20 µl. Ligations for cohesive ends were performed at 14°C and blunt ends at 6-10°C for 12

to 18 hours. Following incubation, ligations were ethanol precipitated (0.5 µl tRNA at 10 mg/ml was added prior to precipitation to increase the concentration of DNA in order to prevent it's loss) washed in 70% (v/v) ethanol, dried and resuspended in 5 µl of sterile nanopure water and transformed.

2.11.6. Phenol:Chloroform Extraction.

An equal volume of phenol:chloroform:isoamyl alcohol (1 part buffer-saturated phenol with Tris-HCl, pH 7.5, 1 part chloroform:isoamyl alcohol (24:1)) was added to the DNA and vortexed for 1 minute to form a suspension. The suspension was centrifuged at 13,000 rpm for 2 minutes in a Micro Centaur bench-top microfuge. The upper aqueous phase was transferred to a fresh tube and retained and the step repeated. To remove residual phenol, an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phase, vortexed for 1 minute and centrifuged as before. The final upper aqueous phase was transferred to a fresh tube.

2.11.7. Ethanol Precipitation of DNA.

DNA was precipitated from the final aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol; the DNA was incubated at –20°C for 30 minutes. The DNA was recovered by centrifugation at 13,000 rpm for 10 minutes in a Micro Centaur microfuge. The resulting pellet was washed in 70% (v/v) ethanol to remove residual salt, dried at room temperature and dissolved in TE buffer.

2.12. Southern Blot Analysis.

Transfer of DNA from agarose gel to nylon membrane was performed as described by the method of Southern (1975) using the following reagents:

Depurination:	0.25 M HCl.
Denaturation:	1.5 M NaCl, 0.5 M NaOH.
Neutralizing:	1.5 M NaCl, 0.5 M Tris-HCl (pH 7.0).
20X SSC:	3 M NaCl, 0.3 M Trisodium citrate (pH 7.0).

50X Denhardt's solution: 1% (w/v) Bovine serum albumin (BSA), 1% (w/v) Ficoll (mol. wt 400,000), 1% (w/v) Polyvinylpyrrolidone (mol. wt 400,000). Aliquoted and stored at –20°C.

2.12.1. DNA Transfer to a Nylon Membrane using Southern Blotting.

Agarose gel electrophoresis was used to separate DNA samples as described (section 2.11.1). After electrophoresis, the DNA bands were photographed with a fluorescent ruler alongside the gel. The gel was rinsed in distilled water and depurinated by soaking the gel in 5 to 10 times the gel volume using 0.25 M HCl solution. The gel was soaked for 30 minutes on a shaking platform set at low speed. The depurination solution was discarded and the gel rinsed in distilled water. The appropriate volume of denaturation solution (5 to 10 times the gel volume) was added and incubation continued at room temperature with shaking for 30 minutes. The solution was poured off and the gel was rinsed in distilled water. The appropriate volume of neutralizing solution (5 to 10 times the gel volume) was added and incubated at room temperature for 15 minutes with shaking. This step was repeated using fresh neutralizing solution. The gel was finally washed in distilled water.

To prepare the blotting apparatus, 20X SSC was added to a container to form a reservoir. Three sheets of pre-wetted Whatman 3MM paper were used to cover a flat platform, the ends of the 3MM paper dipped into the 20X SSC reservoir and served as a wick. The 3MM paper was smoothed to remove trapped air bubbles. The gel was placed on the saturated 3MM paper followed by the nylon membrane (Hybond-N or Hybond-N+, Amersham International PLC) pre-wetted in 20X SSC; any trapped air bubbles were removed. Nescofilm was placed around the edges of the gel so that no part of the nylon membrane could come into direct contact with the 3MM paper wick. On top of the nylon membrane three sheets of pre-wetted (20X SSC) 3MM paper, 5 sheets of dry 3MM paper and a stack of paper towels to a thickness of 4 cm was placed. Finally a 500 g weight was placed on top of the stack and capillary transfer was performed overnight. The apparatus was disassembled after transfer and the nylon membrane placed DNA-side up on a few pieces of 3MM paper soaked in 0.4 M NaOH for 30 minutes. The membrane was washed in 5X SSC, air-dried and wrapped in Saran Wrap (Dupont) and stored at 4°C. Or after disassembly of the apparatus the nylon membrane was placed

DNA-side down on a UV transilluminator and crossed-linked for the time recommended by the manufacturer.

2.12.2. Labelling of the DNA Probe for DNA Hybridisation.

The Ready To Go™ DNA Labelling Kit (-dCTP) from Pharmacia was used to radioactively label DNA. The Reaction Mix in the tube contains a lyophilised pellet of dATP, dGTP, dTTP, klenow fragment and random oligo-deoxyribonucleotides, primarily 9-mers. The Reaction Mix was reconstituted by adding 20 µl of sterile nanopure water and hydrating at room temperature. Purified DNA extract at approximately 100 ng was added to sterile nanopure water to a final volume of 20 µl. The DNA was denatured by boiling at 95°C-100°C for 5 minutes and then immediately cooled on ice for 5 minutes, followed by a pulse-spin in a Micro Centaur bench-top microfuge. The denatured DNA was added to the reconstituted Reaction Mix, mixed by gentle pipetting up and down and given a quick pulse-spin. [α-^{32}P]dCTP at 10 µCi (obtained from Amersham International at a specific activity of 3000 Ci/mmol) was added to the tube and incubated at 37°C for 15 minutes. The tube was boiled at 95°C-100°C for 2-3 minutes and then immediately cooled on ice for 2-3 minutes, given a quick pulse-spin and was ready to use.

2.12.3. Hybridisation of Southern Blot Membrane.

2.12.3.1. Prehybridisation, Hybridisation and Washing.

For prehybridisation, 25 ml of a standard aqueous hybridisation solution was prepared. Salmon sperm was denatured by heating to 100°C for 5 minutes and immediately placed on ice to chill. Aqueous hybridisation solution consisted of 5X SSC, 5X Denhardt's solution, 0.5% (w/v) SDS and salmon sperm at 100 µg/ml. The membrane was incubated at 65°C for at least 60 minutes in the pre-warmed prehybridisation solution with constant shaking. The radiolabelled probe prepared earlier (section 2.12.2.) was added to the prehybridisation solution and incubated for 65°C for at least 12 hours.

Following hybridisation, the membrane was washed in 30 ml of 2X SSC, 0.1% (w/v) SDS at room temperature for 10 minutes. This step was repeated. The solution was replaced with 1X SSC, 0.1% (w/v) SDS and incubated at 65°C for 10 minutes. For high

stringency washes a further wash was performed using 0.1X SSC, 0.1% (w/v) SDS and incubating at 65°C for 10 minutes. The membrane was air-dried to remove excess liquid and wrapped in Saran Wrap (Dupont) to prevent drying out. Membranes were placed in an autoradiograph cassette and exposed to Cronex (Dupont) film for 6 to 24 hours depending on the intensity of the hybridisation signal. Films were processed in an Agfa-Geveart automatic film processor.

2.12.4. Membrane Stripping for Repeated Hybridisation.

To remove radiolabelled probe from the membrane, 0.5% (w/v) SDS was boiled and poured over the membrane and allowed to cool to room temperature. This procedure was repeated three times. The membrane was air-dried, wrapped in Saran Wrap (Dupont) and checked using autoradiography to see if the probe had been removed. The membrane was prehybridised and hybridised with the new probe.

2.13. Dot Blotting DNA: Manual Application to a Nylon Membrane.

The nylon membrane was marked lightly to form a grid to guide subsequent sample application. No pre-wetting step was required for nylon membranes. To the DNA, 2 µl of salmon sperm at 10 µg/ml and 20X SSC was added to give a final concentration of 6X SSC. The samples were incubated in a boiling water bath for 5 minutes to denature the DNA and immediately chilled on ice. The tubes were spun for 10 seconds to ensure that the entire sample collected at the bottom of the microfuge tube and the samples were kept on ice. The samples were applied in approximately 2 µl aliquots to the membrane to concentrate the hybridisation signal. Samples were applied repeatedly, allowing the membrane to dry between the applications. The DNA was fixed to the membrane by UV cross-linking. The membrane was placed DNA-side down on a UV transilluminator and crossed-linked for the time recommended by the manufacturer. The membrane was wrapped in Saran Wrap (Dupont) and stored at room temperature or hybridised (section 2.12.3.1).

2.14. RNA Techniques.

2.14.1. Extraction of RNA from *Escherichia coli.*

Extraction of RNA from *E. coli* was performed as described by the method of Ausubel and others (1992) using the following reagents:

Protoplasting Buffer:	15 mM Tris-HCl (pH 8.0), 0.45 M Sucrose (filter sterilised), 8 mM EDTA.
Lysis Buffer:	10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM Trisodium citrate, 1.5 % (w/v) SDS.
Lysozyme:	50 mg/ml in protoplasting buffer.
Saturated NaCl:	40 g NaCl, 100 ml Distilled water, 100 µl DEPC.

10 ml of an overnight culture was diluted 1:100 in 10 ml Luria broth and grown to mid-exponential phase (OD_{600nm} 0.4-0.5) at 37°C with shaking at 200 rpm. The 10 ml culture was transferred to centrifuge tubes and centrifuged at 2500g for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1ml protoplasting buffer. The solution was transferred to microfuge tubes, 80 µl of lysozyme at 50 mg/ml in protoplasting buffer was added to the tube and incubated on ice for 15 minutes. The protoplasts were centrifuged for 15-20 seconds in a Micro Centaur bench-top microfuge, the supernatants discarded and the resulting pellet resuspended by gently vortexing in the remaining 40-50 µl of protoplasting buffer. 500 µl of lysis buffer and 3% (v/v) DEPC were added to the solution to lyse the protoplasts, followed by incubation at 37°C for 5 minutes. The solution was transferred to ice and 250 µl of saturated NaCl was added, mixed gently and incubated on ice for 15 minutes. The solution was centrifuged at 13,000g for 10 minutes at 4°C and the supernatant transferred to fresh tubes. To each tube, 400 µl of phenol:chloroform:isoamyl alcohol (24:24:1) was added, vortexed vigorously and centrifuged at 13,000 rpm for 5 minutes. 80% of the upper aqueous phase was transferred to fresh tubes and the RNA precipitated by the addition of 2.5 volumes of absolute ethanol and incubated at –20°C overnight. The RNA was recovered

by centrifugation at 13,000 rpm for 30 minutes in a Micro Centaur bench-top microfuge. The resulting pellet was washed in 70% (v/v) ethanol, dried at room temperature and dissolved in 50 µl of DEPC treated nanopure water. To assess the quality and yield of the RNA, 1 µl was run against *E. coli* RNA standard on a TAE agarose gel stained with ethidium bromide. The RNA concentration was estimated at 5 µg/µl.

2.14.2. Extraction of RNA from Mycobacteria.

RNA extraction from mycobacteria was based on the method of Mangan and others (1997). The HYBAID RECOVERY™ RiboLyser™ was used in the extraction of RNA from mycobacteria using the following reagents:

Reagent A:	9.6 ml Divolab No.1 (Diversey Ltd), 24 ml 50 mM Sodium acetate (pH 4.0), 66.4 ml RNase-free water.
Reagent B:	Water saturated phenol equilibrated with 50 mM Sodium acetate (pH 4.0). To 100 ml of water-saturated phenol 50 ml RNase-free water was added, mixed thoroughly and incubated overnight at 4°C. 50 ml of the solution was added to a clean bottle, 16.6 ml 200 mM sodium acetate (pH 4.0) was added, mixed thoroughly and incubated overnight at 4°C.
Reagent C:	Chloroform isoamyl alcohol 24:1.
Reagent D:	0.3 ml 3M Sodium acetate (pH 4.0), 49.7 ml Isopropanol.
Reagent E:	70 ml absolute ethanol, 30 ml RNase-free water.

Bacteria were grown to mid-exponential phase in 7H9 broth at 37°C to obtain 10^9 cells; 75 ml culture was centrifuged at 6,000 rpm for 15 minutes in a Heraeus Christ centrifuge and the supernatant discarded. The cells were washed in 2 ml of 0.5% (v/v) Tween 80 solution (a critical step to remove some ingredients of growth medium that can effect the efficiency of extraction). The culture was aliquoted equally into two fresh

tubes and centrifuged at 13,000 rpm for 10 minutes at room temperature in a Micro Centaur bench-top microfuge. The resulting pellet was resuspended in 200 µl of Rnase free distilled water. To the HYBAID RiboLyser™ matrix blue cap tube containing acid-washed 0.1 mm silica/ceramic beads, 500 µl of detergent solution (Reagent A), 500 µl of acid phenol (Reagent B) and 100 µl of chloroform:isoamyl alcohol (24:1) (Reagent C) were added. To this, 200 µl of resuspended bacterial pellet was added and placed in the HYBAID RiboLyser™ instrument. The tubes were immediately processed in the shaker for 45 seconds at a speed rating of 6.5 (top speed), removed from the machine and placed on ice for 10 minutes to cool down.

Cell debris was removed by centrifugation in a Micro Centaur bench-top microfuge at 13,000 rpm for 10 minutes, followed by the removal of the upper aqueous phase into a fresh tube. To this, 300 µl of chloroform:isoamyl alcohol (24:1) (Reagent C) was added, vortexed and centrifuged at 13,000 rpm for 2 minutes to separate the phases. The upper aqueous phase was transferred to a fresh tube containing 500 µl of isopropanol solution (Reagent D), mixed and incubated on dry ice for 10 minutes. The precipitated RNA pellet was centrifuged at 13,000 rpm for 15 minutes in a Micro Centaur microfuge, the supernatant removed and the pellet washed once in 70% (v/v) ethanol (Reagent E). The tube was centrifuged briefly to remove excess liquid, dried and dissolved in 100 µl of Rnase free distilled water (Reagent F). RNA samples were aliquoted and stored at – 70°C. To assess the quality and yield of the RNA, 1 µl was run against *E. coli* RNA standard on a TAE agarose gel stained with ethidium bromide. The RNA concentration was estimated at 1 µg/µl.

2.14.3. Agarose Gel Electrophoresis of RNA.

Denaturation of RNA was performed as described by the method of Murray and others (1994). Transfer of RNA from agarose gel to nylon membrane was performed as described by the method of Bresser and Gillespie (1983). Agarose gel electrophoresis was done to separate RNA bands using the following reagents:

GFM Buffer:	78% (v/v) Formamide, 1.1 M Glyoxal, 0.06X MOPS (3-(N-morpholino) propanesulphonic acid) (pH 7.0).

Formamide:	Formamide was deionised by adding 2 g per 100 ml of Bio-Rad AG501-X8 (Δ) bed resin and stirring for 1 hour at room temperature. The resin was allowed to settle, formamide was filter sterilised, aliquoted and stored at -70°C.
Glyoxal:	40% (v/v) aqueous solution (Sigma) was heated to 50°C to 60°C until all precipitate had dissolved. Deionisation repeated as with formamide. Glyoxal was aliquoted and stored at -70°C.
10X MOPS (pH 7.0):	200 mM MOPS, 50 mM Sodium acetate, 1 mM EDTA.
RNA Sample Buffer:	40% (v/v) Deionized formamide, 50% (v/v) Glycerol, 1X MOPS (pH 7.0), 0.025% (w/v) Xylene cyanol, 0.025% (w/v) Bromophenol blue.

Agarose (SeaKem) at 1.1% (w/v) was dissolved in 1X MOPS by heating for several minutes. RNA samples were thawed on ice, 1 to 3 volumes of GFM buffer was added to each aliquot of 5 μg of RNA and to 3 μl of RNA size marker and heated at 55°C for 20 minutes. 2 μl of RNA sample buffer was added to each sample and spun for 10 seconds to ensure that the sample collected at the bottom of the tube. The samples were loaded into the gel and run using 1X MOPS as the running buffer. The gel was run until the blue marker dye reached one inch away from the end of the gel. The gel was stained for approximately 10 minutes in 1X MOPS containing 0.5 μg/ml of ethidium bromide. After electrophoresis the RNA bands were photographed with a fluorescent ruler alongside the gel, followed by transfer of RNA samples to a nylon membrane.

2.14.4. Northern Blot Analysis.

Northern blot analysis of RNA was performed as in the method of Southern blot analysis, prehybridisation, hybridisation and washing (section 2.12). In addition, after transfer of RNA samples to the nylon membrane, the membrane was stained with

methylene blue to detect and visualise RNA. This method did not affect any subsequent procedures.

2.15. Bioluminescent Assay and Alamar Blue Assay.

The following experiments were performed to compare the use of alamar blue and bacterial luciferase for the high-throughput screening of potential antimycobacterial compounds.

2.15.1. Chemicals.

Amikacin, pyrazinamide and rifampicin were made to a stock concentration of 5 mg/ml and filter sterilised (0.2 μm: Gelman Sciences). Decanal was prepared fresh at 1% (v/v) in absolute ethanol. Alamar blue solution was freshly prepared by dissolving one resazurin tablet in 5 ml sterile distilled water.

2.15.2. Media and Growth Conditions.

Mycobacterial cultures were grown in 7H9 broth. *Mycobacterium smegmatis* (pSMT1) was inoculated into 10 ml of 7H9 broth and incubated at 37°C with shaking at 225 rpm for approximately 48 hours in the presence of 50 μg/ml of hygromycin B. *Mycobacterium aurum* (pPA3) was grown in 10 ml 7H9 broth as static cultures in air at 37°C for 5 days in the presence of 20 μg/ml of kanamycin.

2.15.3. Minimum Inhibitory Concentration Determination and Culture Preparation.

The minimum inhibitory concentrations (MIC) for *M. smegmatis* and *M. aurum* of amikacin, pyrazinamide and rifampicin were determined in transparent 96-well microtitre plates (Nalgene Nunc International). The drugs were prepared immediately before use. Serial doubling dilutions of each in 100 μl in 7H9 broth was added to wells to achieve final concentrations ranging from 250 to 0.008 μg/ml. Mycobacterial cultures, grown for 48 hours and 5 days for *M. smegmatis* and *M. aurum* respectively, were sonicated (Ultrasonic Engineering, UK) on ice at 50W for 3 bursts of 5 seconds to disrupt clumps of bacteria and then diluted in the same medium to an OD_{550nm} of 0.125 (yielding an inoculum of approximately 10^5 cfu/ml). The microtitre plates were placed

in a humidified container and incubated at 37°C for 24 hours for both the bioluminescent assay and the alamar blue assay.

2.15.4. Bioluminescent Assay.

After incubation for 24 hours, cultures for the bioluminescence assay were transferred from the 96-well microtitre plates into wells of a black microtitre plate (ThermoQuest Scientific Equipment Group Ltd, Hampshire, UK) and the bioluminescence measured in a luminoskan luminometer (Labsystems Ltd, Hampshire, UK). 10 µl of fresh 1% (v/v) decanal in ethanol was dispensed automatically into the test and control wells, mixed for 1 second and bioluminescence was measured for a duration of 15 seconds at 37°C. Bioluminescence was expressed as the number of relative light units (RLU) detected within this period.

2.15.5. Alamar Blue Assay.

For the alamar blue assay, after 24 hours incubation 20 µl of fresh alamar blue solution was added to each well (BDH, U.K). The plates were then incubated for an additional 24 hours at 37°C and the results recorded. MICs were assessed visually and fluorometrically using 530nm excitation and 590nm emission wavelengths (Perkin Elmer). Visual results were determined as pink coloration in the wells (no inhibition of growth) or blue (inhibition of growth). The MIC was determined as the lowest concentration of drug giving a blue colour. Occasionally, the well colour was purple before the next lowest drug dilution giving a pink reaction. In this instance, the next highest well giving a clear blue after the purple reaction was recorded as the MIC.

2.15.6. Viable Counting of Mycobacteria.

Colony-forming units (CFUs) were determined using the method of Miles and Misra (1938). In a 96 well microtitre plate 20 µl of the sonicated mycobacterial suspension was added to 180 µl of 7H9 broth and serially diluted to a dilution of 10^6. 50 µl of each dilution were pipetted onto one sixth of an agar plate. Luria agar was used for *M. smegmatis* and 7H11 agar for BCG and *M. tuberculosis*. The plates were left to dry, sealed with Nescofilm and placed in Petri-dish bags for incubation. The plates were incubated at 37°C for approximately 2 days for *M. smegmatis* and 3 to 4 weeks for BCG and *M. tuberculosis* or until colonies were visible on the plates. Approximately 30 to

300 colonies were counted on a particular dilution using a colony counter (Anderman and Company Ltd., U.K.). The numbers of colony forming units per millilitre were then calculated to give the number of viable bacteria in the original suspension.

2.16. Macrophage Culture and Infection.

These experiments were done to assess the activity of antimycobacterial drugs against mycobacteria within macrophages.

2.16.1. Tissue Culture Reagents.

All reagents were obtained from Gibco-BRL (c/o Life Technologies, Paisley, U.K.) unless otherwise stated. Hanks balanced salt solution, calcium and magnesium free, without phenol red was buffered with 25 mM Hepes, pH 7.4 (HBSS-Hepes). RPMI 1640 tissue culture medium was supplemented with 1 mM L-glutamine and with either 10% (v/v) or 1% (v/v) heat inactivated foetal bovine serum (HIFBS). RPMI 1640 medium plus HIFBS was incubated at 37°C and 5% (v/v) CO_2 overnight to assess sterility of the medium prior to use. Foetal bovine serum (HIFBS) was heat inactivated at 56°C for 30 minutes followed by filter sterilising through a 0.2μm acrodisc (Gelman). All tissue culture media were stored at 4°C until required. Trypsin-EDTA Solution consisted of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA in Hanks balanced salt solution, calcium and magnesium free, without phenol red (HBSS). Filter sterilised through a 0.2 μm acrodisc (Gelman) and stored at – 4°C.

2.16.2. Tissue Culture Plasticware.

Tissue culture flasks, 24 well tissue culture plates and 96 well microtitre plates were obtained from Nunc (c/o Life Technologies, Paisley, U.K.).

2.16.3. Growth and Maintenance of Murine Macrophage Cell Line, J774.

Growth and maintenance of murine macrophage cell line J774 was based on the method of Ralph *et al.*, 1975. The J774 cells were recovered from liquid nitrogen stocks in the Department of Microbiology and Immunology, University of Leicester (the cells originally obtained from the European Collection of Animal Cell Culture, U.K.). J774s were grown in RPMI 1640 medium supplemented with 10% (v/v) HIFBS at 37°C and 5% (v/v) CO_2. When a confluent monolayer had formed on the surface of the tissue

culture flask, the cells were subcultured. The medium was removed; the cells were washed twice in 10 ml of HBSS-Hepes and 2 ml of trypsin-EDTA solution was added to the monolayer. The monolayer was incubated at 37°C and 5% (v/v) CO_2 for approximately 5 minutes, after which the cells were removed from the surface by sharp tapping on the flask. 20 ml of fresh RPMI 1640 medium plus HIFBS was added to the flask, swirled and transferred to a falcon centrifuge tube and centrifuged at 1,000 rpm for 5 minutes in a Centaur 2 MSE centrifuge to remove traces of trypsin-EDTA. The medium was removed and 1 ml fresh RPMI 1640 medium plus HIFBS was added and the cells pipetted gently to separate clumps. 300 µl of the cell suspension was added to 10 ml RPMI 1640 medium plus HIFBS into a clean tissue culture flask and the cells were incubated at 37°C and 5% (v/v) CO_2.

2.16.4. Trypan Blue Exclusion Assay to Determine Viable Cell Number.

To count numbers of viable macrophages, 20 µl of the cell suspension was added to 40 µl of 0.2% (v/v) trypan blue in Hanks balanced salt solution, calcium and magnesium free without phenol red. 20 µl of this solution was then transferred to a chamber of the haemocytometer (Improved Neubauer, Depth 0.1 mm, 1/400 mm^2, Weber, U.K.) and the cells were counted. Viable cells remained unstained and white in colour and dead cells stained blue.

2.16.5. Preservation of J774 Cells in Liquid Nitrogen.

J774 cells were deposited in liquid nitrogen to preserve a stock (O'Brien, 1995). A confluent monolayer of J774s were removed from the surface of the flask as described (section 2.16.3.) and the number of viable cells counted with a haemocytometer (section 2.16.4.), the concentration was adjusted to $1x10^7$ cells/ml in RPMI 1640 plus 10% (v/v) HIFBS and 20% (v/v) glycerol or in HIFBS plus 5% DMSO. The cell suspension was aliquoted in 1 ml volumes into sterile cryotubes and placed within a cryo 1°C freezing container (Nalgene™, U.K.) containing isopropanol to achieve a 1°C per minute rate of cooling. The container was placed at –70°C overnight to allow the cells to freeze and the cryotubes were then transferred to the liquid nitrogen container for long-term storage.

2.16.6. Recovery of J774 Cells from Liquid Nitrogen.

The cells were recovered from liquid nitrogen by rapid thawing at 37°C in a water bath. Once thawed, 1 ml of cells were added to 9 ml RPMI 1640 medium plus 10% (v/v) HIFBS and centrifuged at 1,000 rpm for 5 minutes in a Centaur 2 MSE centrifuge to remove traces of glycerol or DMSO. The supernatant was discarded, 10 ml of fresh RPMI 1640 medium plus 10% (v/v) HIFBS was added and transferred to a clean tissue culture flask. The cells were incubated at 37°C and 5% (v/v) CO_2. After several days the cells began to grow at a normal rate.

2.16.7. Preparation of Mycobacteria.

Mycobacteria were grown for 2 days (*M. smegmatis*) or 5 days (*M. aurum*) in 7H9 broth, harvested by centrifugation at 1,000g for 10 minutes and then washed twice in HBSS-Hepes. The cell pellets were resuspended in 1 ml of HBSS-Hepes and sonicated on ice for three 5 second bursts at 50W (Ultrasonic Engineering, U.K.) to disrupt the clumps of bacteria. The mycobacteria were counted microscopically using the haemocytometer and were then diluted in RPMI 1640 medium plus 1% (v/v) HIFBS to the specified working concentration.

2.16.8. Preparation of Monolayer.

The method of preparing and infecting macrophages was based on Khor and others (1986). To prepare the monolayer, J774s were removed from the tissue culture flask and counted using the haemocytometer. Trypan blue exclusion assay was used to determine viability as described (section 2.16.4). The J774s were prepared as described (section 2.16.3.) and $3x10^7$ cells per well in a volume of 350 µl were pipetted into a 24 well tissue culture plate. The cells were incubated for 24 hours at 37°C and 5% (v/v) CO_2 allowing for adherence of the cells to the surface of the wells. After 24 hours, non-adherent cells were removed by washing once with HBSS-Hepes. The resulting macrophage monolayer was cultured in RPMI 1640 medium plus 1% (v/v) HIFBS to reduce cell proliferation.

2.16.9. Infection of Monolayer.

Mycobacteria were prepared as described in section 2.16.7. The mycobacteria were diluted in RPMI 1640 medium plus 1% (v/v) HIFBS to obtain a 1:1 ratio of

Mycobacterium to macrophage in the monolayer of the 24 well tissue culture plate. 350 µl of medium containing $3x10^7$ bacteria (including the appropriate antibiotic for maintenance of the plasmid) were gently added into the wells of the 24 well tissue culture plate containing the adherent J774s and incubated for 4 hours at 37°C and 5% (v/v) CO_2 to allow phagocytosis. The supernatant was aspirated and set-aside to establish the number of bacteria remaining unattached, whilst the monolayer was washed four times in HBSS-Hepes to remove unphagocytosed mycobacteria. Fresh RPMI 1640 medium plus 1% (v/v) HIFBS with or without antimycobacterial drug were added to the macrophages, including the appropriate antibiotic for maintenance of the plasmid. Macrophages were incubated for 24 hours at 37°C and 5% (v/v) CO_2.

2.16.10. Antimycobacterial Activity Estimated from Expression of *lux* and Viable Counts.

The supernatants from each well of the 24 well tissue culture plate were removed and set-aside. The macrophages were removed from the wells of the plate by addition of 350 µl of 1% (w/v) saponin in HBSS-Hepes. 35 µl of 10% (w/v) saponin in HBSS-Hepes was added to the supernatants, including that set-aside after the 4 hour infection of the monolayer. The 24 well tissue culture plate and supernatants were incubated at 37°C for 20 minutes or until the macrophages had completely lysed and then aspirated by pipetting up and down. Cell lysis was checked microscopically using a Nikon TMS (Japan) inverted microscope. Cell lysates and supernatant were briefly sonicated on ice for three 5 second bursts at 50W (Ultrasonic Engineering, U.K.) to ensure complete cell lysis and disruption of any bacterial clumps. 300 µl of lysates were added to 96 well black microtitre plates and bioluminescence measured as described in section 2.15.4. Viable counts were performed to estimate the number of extracellular and intracellular bacteria as described in section 2.15.6.

2.17. Acid Tolerance Response and Acid Adaptation of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG.

To demonstrate an acid tolerance response in *M. smegmatis* and *M. bovis* BCG, the adaptive and lethal pH for the mycobacteria had to be determined. The survival of mycobacteria at several different pHs was tested using 7H9 broth acidified with 2 M hydrochloric acid (HCl) or 2 M phosphoric acid (H_3PO_4). 2 M NaOH was used to adjust

7H9 broth to pH 7.6. Cultures were centrifuged and the bacterial pellets resuspended in 7H9 broth acidified earlier, in order that the pH of the medium could be accurately measured. To prevent further stress on the mycobacteria non-sonicated cultures were used throughout the experiments. All media were pre-warmed to 37°C before use and pH of the media was measured after the experiments.

2.17.1. Survival of *M. smegmatis* Over a Range of pH.

A 10 ml starter culture of *M. smegmatis* mc^2155 was grown in 7H9 broth pH 6.6-6.8 (standard pH) at 37°C overnight with shaking at 200 rpm. Fresh 10 ml 7H9 broth at pH 7.6 was inoculated with the overnight culture and incubation continued overnight with shaking at 200 rpm. 1 ml of culture was sonicated on ice for three 5 second bursts at 50W (Ultrasonic Engineering, U.K.) to disrupt the clumps of bacteria. The mycobacteria were diluted and counted microscopically using the haemocytometer. $1x10^6$ bacteria/ml of non-sonicated mycobacteria were centrifuged at 5,000 rpm for 10 minutes at room temperature in a Heraeus Christ centrifuge and resuspended in 7H9 broth at pH 7.6 in a volume of 50µl. Each 50µl when added to 7H9 broth at the following pHs, 7.6, 6.0, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5, contained approximately $1x10^6$ bacteria/ml. Cultures were incubated at 37°C with shaking at 200 rpm. 1 ml samples of each culture were removed at 0, 2, 4 and 6 hours, sonicated and viable counts performed as described (section 2.15.6). 2 M hydrochloric acid (HCl) or 2 M phosphoric acid (H_3PO_4) were used to pH the media.

2.17.2. Testing *M. smegmatis* for an Acid Tolerance Response with Hydrochloric Acid as the Acidulant.

The method used to investigate the acid tolerance response of *M. smegmatis* was based on Foster and Hall (1990). Cultures of *M. smegmatis* were grown overnight and 7H9 broth at pH 7.6 was inoculated with $1x10^6$ bacteria/ml as described (section 2.17.1). The cultures were incubated for 14 hours at 37°C with shaking at 200 rpm to obtain $1x10^8$ bacteria/ml. The cultures were harvested by centrifugation at 2,500 rpm for 10 minutes at room temperature in a Mistral 3000i centrifuge. The supernatants were removed and the cell pellets resuspended in 7H9 broth at the control pH of 7.6 and adaptive pH of 4.5 or 4.0, using the same volume used to grow the mycobacteria. Cultures at pH 7.6 were incubated for a further 2 hours and cultures at pH 4.5 or 4.0 were incubated for 4 hours.

This allowed one doubling of the bacteria, to obtain approximately $2x10^8$ bacteria/ml. After incubation cultures were centrifuged at 2,500 rpm for 10 minutes at room temperature in a Mistral 3000i centrifuge and the cell pellets resuspended in 7H9 broth at the lethal pH of 2.5 using 2 M hydrochloric acid (HCl). 1 ml samples of each culture were removed at 0, 1, 2, 3, 4 and 6 hours, sonicated and viable counts performed as described (section 2.15.6). In addition, 300 µl samples of culture were taken to measure bioluminescence as described (section 2.15.4).

2.17.3. Testing *M. smegmatis* for an Acid Tolerance Response with Phosphoric Acid as the Acidulant.

The experiment was repeated exactly as that described for hydrochloric acid (section 2.17.2.) except 2 M phosphoric acid was used to acidify the 7H9 medium. An adaptation pH of 5.0 or 4.5 and a lethal pH of 2.5 were tested.

2.17.4. Testing for Expression of *lux* in *M. smegmatis* at the 'Adaptive' pH with Hydrochloric Acid as the Acidulant.

To measure the expression of *lux* at the adaptive pH, samples of cultures were taken at various time points and measured for the production of light. This method was based on a modification of the method for determining the acid tolerance response of *M. smegmatis*.

The experiment was repeated as described in section 2.17.2., up until inoculation at the adaptive pH. Cultures were resuspended in 7H9 broth at the control pH of 7.6 and adaptive pH of 4.5 or 4.0. The medium was acidified with 2 M hydrochloric acid (HCl). 1 ml samples of culture were removed and measured at times 0, 1, 2, 3, 4, 8, 24, 32 and 48 hours. Cultures were incubated at 37°C between the time points. Samples were sonicated and viable counts performed as described (section 2.15.6). In addition 300 µl samples of culture were taken to measure bioluminescence as described (section 2.15.4).

2.17.5. Testing for Expression of *lux* in *M. smegmatis* at the 'Adaptive' pH with Phosphoric Acid as the Acidulant.

The experiment was repeated exactly as that described for hydrochloric acid (section 2.17.5.) except 2 M phosphoric acid was used to acidify the 7H9 medium. The 7H9 broth was adjusted to pH 5.0 or pH 4.5 for the adaptive pH.

2.17.6. Survival of *M. bovis* BCG Over a Range of pH.

A 10 ml starter culture of BCG was grown in 7H9 broth pH 6.6-6.8 (standard pH) at 37°C stationary for approximately 7 days. 2.5 ml of the 7-day culture was centrifuged at 5,000 rpm for 10 minutes at room temperature in a Heraeus Christ centrifuge and resuspended in 500 µl of 7H9 broth at pH 7.6. 500 µl of the culture was inoculated into 25 ml of 7H9 broth at pH 7.6 to obtain a 1:10 dilution and incubated for 72 hours at 37°C. 1 ml of culture was sonicated on ice for three 5 second bursts at 50W (Ultrasonic Engineering, U.K.) to disrupt the clumps of bacteria. The mycobacteria were diluted and counted microscopically using the haemocytometer. $1x10^6$ bacteria/ml of non-sonicated mycobacteria were centrifuged at 5,000 rpm for 10 minutes at room temperature in a Heraeus Christ centrifuge and resuspended in 7H9 broth at pH 7.6 in a volume of 50µl. Each 50µl when added to 7H9 broth at the following pHs, 7.6, 6.6, 6.0, 5.0, 4.5, 4.0, 3.5, 3.0, and 2.5, contained approximately $1x10^6$ bacteria/ml. Cultures were incubated at 37°C. 1 ml samples of each culture were removed at 0, 6, 24 and 48 hours, sonicated and viable counts performed as described (section 2.15.6). 2 M hydrochloric acid (HCl) or 2 M phosphoric acid (H_3PO_4) were used to pH the media.

2.17.7. Testing BCG for an Acid Tolerance Response with Hydrochloric Acid as the Acidulant.

Investigation of an acid tolerance response in BCG was developed from the method for determining an acid tolerance response in *M. smegmatis*. All BCG cultures were grown in $25cm^2$ or $50cm^2$ tissue culture flasks to optimise aeration. A 10 ml starter culture of BCG was grown in 7H9 broth pH 6.6-6.8 (standard pH) at 37°C stationary for 7 days. The 7-day culture was inoculated into 7H9 broth pH 7.6 with $1x10^6$ bacteria/ml as described (section 2.17.6). The cultures were incubated at 37°C stationary for 6 days to obtain $1x10^8$ bacteria/ml. Cultures were harvested by centrifugation at 2,500 rpm for 10 minutes at room temperature in a Mistral 3000i centrifuge. The supernatants removed

and the pellets resuspended in 7H9 broth at the control pH of 7.6 and adaptive pH of 6.6 or 5.0, using the same growth volume. All cultures were incubated for a further 24 hours to allow one doubling of the bacteria, to obtain approximately $2x10^8$ bacteria/ml. After incubation cultures were centrifuged at 2,500 rpm for 10 minutes at room temperature in a Mistral 3000i centrifuge and the cell pellets resuspended in 7H9 broth at the lethal pH of 3.0 using 2 M hydrochloric acid (HCl). 1 ml samples of each culture were taken at 0, 6, 24 and 48 hours, sonicated and viable counts performed as described (section 2.15.6). In addition, 300 µl samples of culture were taken to measure bioluminescence as described (section 2.15.4).

2.17.8. Testing BCG for an Acid Tolerance Response with Phosphoric Acid as the Acidulant.

The experiment was repeated exactly as that described for hydrochloric acid (section 2.17.7.) except 2 M phosphoric acid was used to acidify the 7H9 medium. A number of pHs were tested for adaptation to increase the chance of finding an acid tolerance response in BCG. Adaptation pHs of 5.5, 5.0, 4.5 or 4.0 and a lethal pH of 3.0 were tested.

2.17.9. Testing for Expression of *lux* in BCG at the 'Adaptive' pH with Hydrochloric Acid as the Acidulant.

To measure the expression of *lux* at the adaptive pH, samples of cultures were taken at various time points and measured for the production of light. This method was based on a modification of the method for determining the acid tolerance response of BCG. The experiment was repeated as in the acid tolerance response of BCG as described (section 2.17.7.) up until inoculation at the adaptive pH. Cultures were resuspended in 7H9 broth at the control pH of 7.6 and adaptive pH of 4.5 or 4.0 using the same growth volume. The medium was acidified with 2 M hydrochloric acid (HCl). 1 ml samples of culture were removed and measured at times 0, 6, 24 and 48 hours. Cultures were incubated at 37°C between the time points. Samples were sonicated and viable counts performed as described (section 2.15.6). In addition 300 µl samples of culture were taken to measure bioluminescence as described section 2.15.4.

2.17.10. Testing for Expression of *lux* in BCG at the 'Adaptive' pH with Phosphoric Acid as the Acidulant.

The experiment was repeated exactly as that described for hydrochloric acid (section 2.17.9.) except 2 M phosphoric acid was used to acidify the 7H9 medium. The 7H9 broth was adjusted to pH of 4.5 or 4.0 for the adaptive pH.

2.18. Growth Curve of *M. bovis* BCG.

A growth curve of BCG at pH 7.6 was done to assess growth at this pH. BCG was grown for 7 days in standard 7H9 broth at 37°C stationary. The culture was inoculated into 7H9 broth at pH 7.6 to give a 1:10 dilution of culture to medium and 1 ml samples were taken at time 0, 24, 48, 72, 96, 144, 168, 192, 216 and 240 hours. The cultures were incubated at 37°C between time points. Samples were sonicated on ice for three 5 second bursts at 50W (Ultrasonic Engineering, U.K.) to disrupt the clumps of bacteria and then the OD_{550nm} was measured and viability determined as described (section 2.15.6).

2.19. Statistical Analysis

Data were analysed for significance with SPSS version 9.0 using Analysis Of Variance (ANOVA) as advised by John Becket (Statistician).

CHAPTER 3: RESULTS.

3.1. Aim.

The aim of the work described in this chapter was to construct an expression vector based on the *lux* operon for use as a reporter system. The following work in this study was done specifically for the development and assessment of a high throughput screening method for the detection of potential antimycobacterial compounds.

3.2. Vector Construction.

3.2.1. Construction of the expression vector, pJS6.

The expression vector pJS6 based on the *lux* operon was constructed for use in mycobacteria (Figure 3.1). The *Escherichia coli-Mycobacterium* shuttle vector pSMT3 was used for the basis of construction for the vector, which consisted of an origin of replication for *E. coli* and mycobacteria and a hygromycin resistance gene. The expression of the *lux* operon would be under control of the *M. bovis* BCG *hsp*60 gene promoter (Stover *et al.*, 1991). The *luxCDABE* genes of *Photorhabdus luminescens* strain Hb (also known as *Xenorhabdus luminescens*) was obtained from pSfi390 (Swift *et al.*, 1995). To construct the expression vector, pJS6, pSfi390 was digested with *Pst* I resulting in the excision of the *luxCDABE* fragment of approximately 6 Kb. Subsequently, pSMT3 DNA was digested with *Pst* I to linearise the DNA and dephosphorylated to prevent recircularisation of the vector DNA. The 6 Kb *Pst* I fragment of *luxCDABE* was ligated into the *Pst* I site of pSMT3. The ligation product was introduced into *E. coli* strain DH5α by electroporation and transformants were selected on 200 μg/ml hygromycin B.

Transformants containing the *luxCDABE* genes were identified by exposing Cronex film to the transformants for several minutes depending on the intensity of the light signal (Figure 3.2. and Figure 3.3). There was no addition of the substrate, decanal. High levels of light were produced by the transformants. Light colonies were identified and chosen for further investigation. DNA was prepared from one colony and to confirm the orientation and presence of the *luxCDABE* fragment in pSMT3 the DNA was digested with either *Eco*R V or *Bst*X I (Figure 3.4). Two constructs were identified, pJS5 and pJS6. Digestion of pJS5 plasmid DNA with *Eco*R V resulted in two fragments of approximate size of <12000 bp and 300 bp (Figure 3.4.A). Digestion of pJS6 plasmid

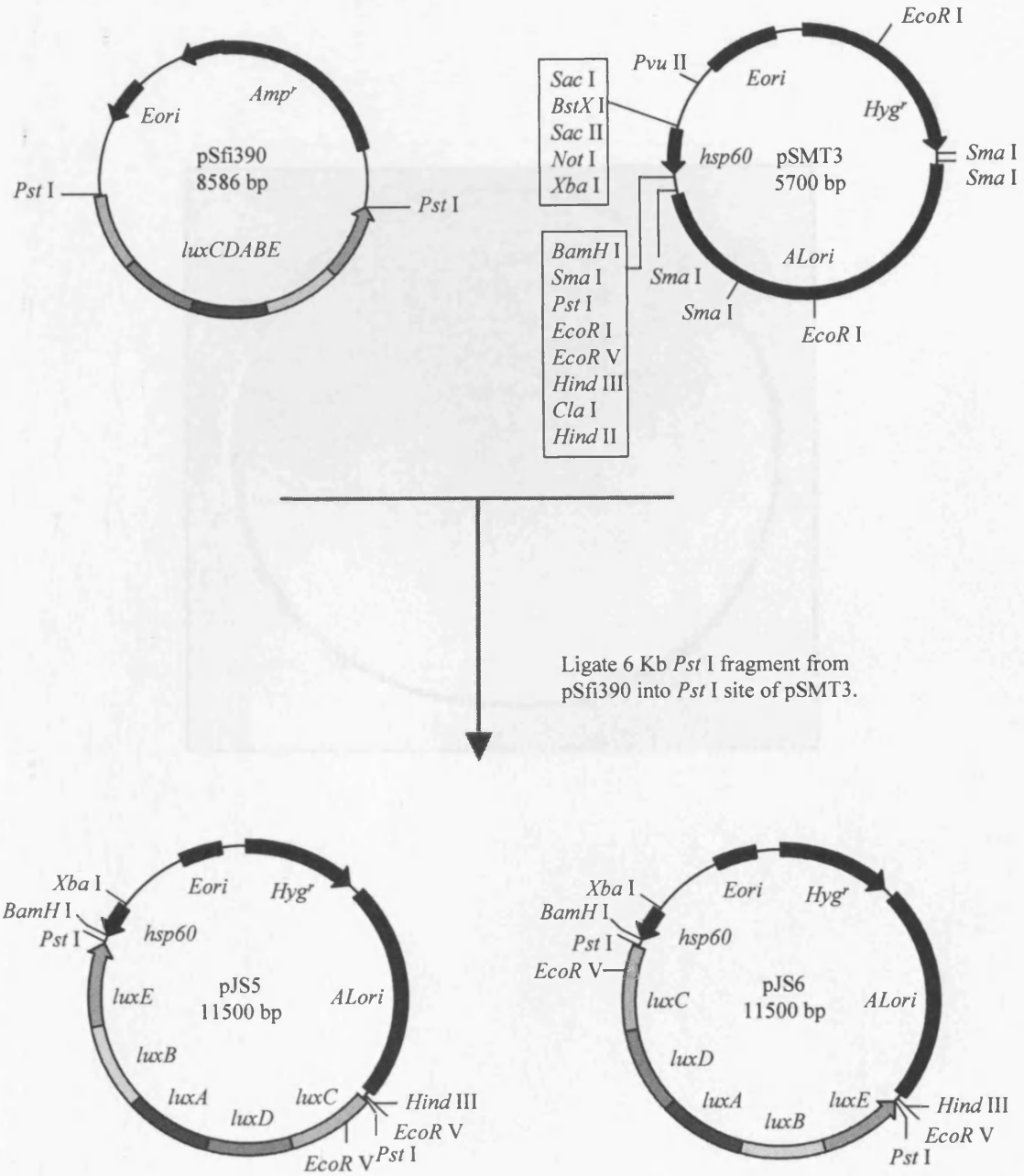


Figure 3.1. Construction of the expression vectors, pJS5 and pJS6.

The *luxCDABE* genes from pSfi390 were digested with *Pst* I and ligated into the *Pst* I site of pSMT3 to produce pJS5 and pJS6. Abbreviations are mycobacterial origin of replication (*ALori*), *Escherichia coli* origin of replication (*Eori*), *M. bovis* BCG promoter (*hsp60*), hygromycin resistance (Hyg^r), ampicillin resistance (Amp^r) and *lux* operon (*luxCDABE*).

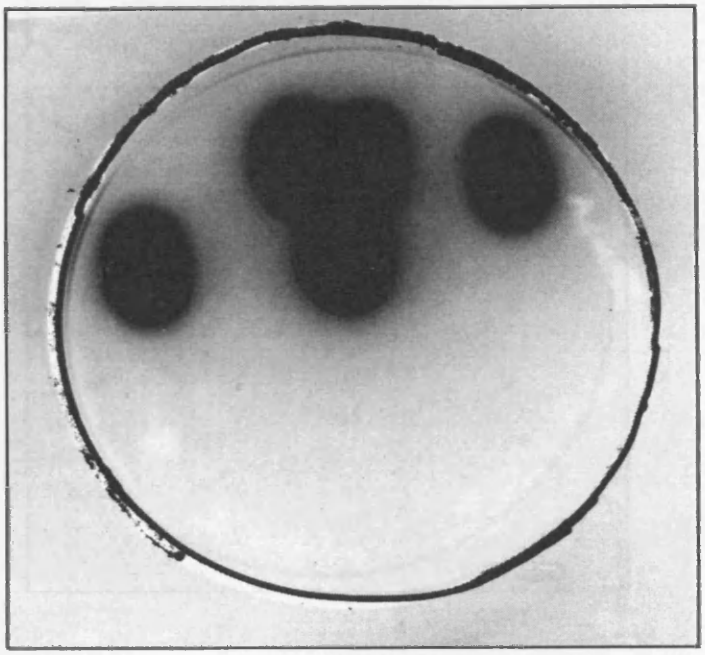


Figure 3.2. The vector, pJS5.

Recombinant *Escherichia coli* were identified as containing pJS5. The detection of light from *E. coli* transformed with pJS5 containing the *luxCDABE* genes was done by exposing Cronex film to transformants in complete darkness for ten minutes. There was no addition of the substrate, decanal. The X-ray film shows colonies that were light (black dots). (Photograph of a Petri-dish).

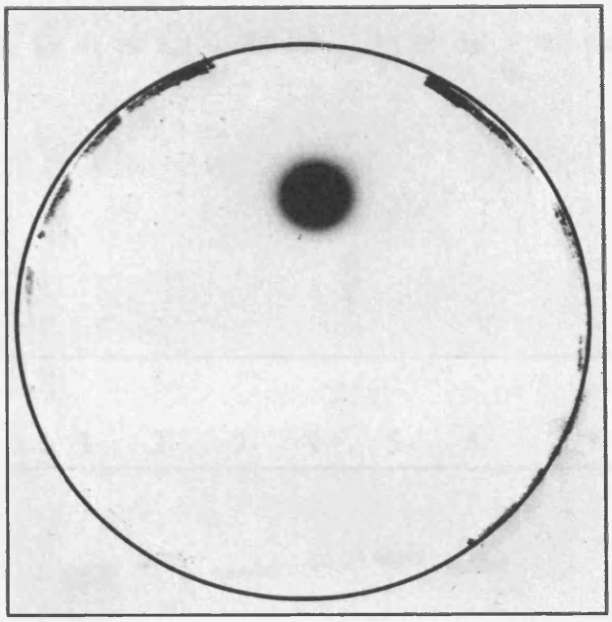


Figure 3.3. The vector, pJS6.

Recombinant *Escherichia coli* was identified as containing pJS6. The detection of light from a single colony of *E. coli* transformed with pJS6 containing the *luxCDABE* genes was done by exposing Cronex film to the transformant in complete darkness for ten minutes. There was no addition of the substrate, decanal. The X-ray film shows a colony that was light (black dot). (Photograph of a Petri-dish).

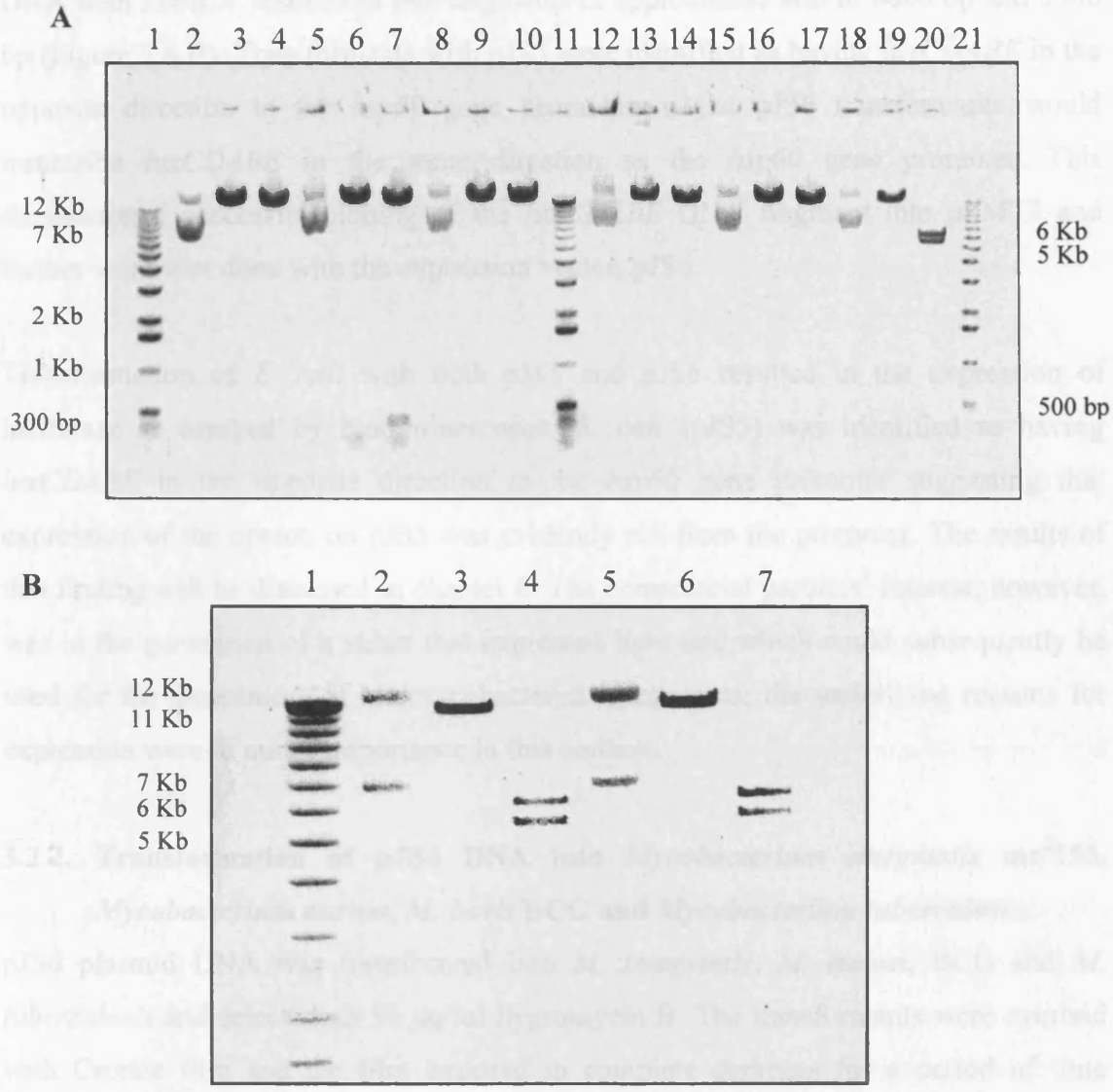


Figure 3.4. Construction of the vectors, pJS5 and pJS6.

To confirm the orientation and presence of the *luxCDABE* fragment in pSMT3 the DNA was digested with either *Eco*R V or *Bst*X I. Two constructs were identified, pJS5 and pJS6. Photograph A: digestion of pJS5 plasmid DNA with *Eco*R V resulted in two fragments of approximate size of <12000 bp and 300 bp (Lanes 4, 7, 10, 14 and 17). Digestion with *Bst*X I resulted in one fragment of approximate size of 12000 bp (Lanes 3, 6, 9, 13 and 16). Lanes 2, 5, 8, 12 and 15 contain undigested DNA. Lanes 1, 11 and 21 contain DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb) and basepairs (bp). Photograph B: digestion of pJS6 plasmid DNA with *Eco*R V resulted in two fragments of approximate size of 6000 bp and 5500 bp (Lanes 4 and 7 (Lane 20 in Photograph A)). Digestion with *Bst*X I resulted in one fragment of approximate size of 12000 bp (Lanes 3 and 6 (Lane 19 in Photograph A)). Lanes 2 and 5 contain undigested DNA (Lane 18 in Photograph A). Lane 1 contains 1 Kb DNA size marker.

DNA with *Eco*R V resulted in two fragments of approximate size of 6000 bp and 5500 bp (Figure 3.4.B). Transformants with pJS5 were identified as having *luxCDABE* in the opposite direction to the *hsp*60 gene promoter, whilst pJS6 transformants would transcribe *luxCDABE* in the same direction as the *hsp*60 gene promoter. This demonstrated successful cloning of the *luxCDABE* DNA fragment into pSMT3 and further work was done with the expression vector, pJS6.

Transformation of *E. coli* with both pJS5 and pJS6 resulted in the expression of luciferase as assayed by bioluminescence. *E. coli* (pJS5) was identified as having *luxCDABE* in the opposite direction to the *hsp*60 gene promoter suggesting that expression of the operon on pJS5 was evidently not from the promoter. The results of this finding will be discussed in chapter 6. The commercial partners' interest, however, was in the generation of a strain that expressed light and which could subsequently be used for the assessment of antimycobacterial compounds; the underlying reasons for expression were of minor importance in this context.

3.2.2. Transformation of pJS6 DNA into *Mycobacterium smegmatis* mc^{2}155, *Mycobacterium aurum*, *M. bovis* BCG and *Mycobacterium tuberculosis*.

pJS6 plasmid DNA was transformed into *M. smegmatis*, *M. aurum*, BCG and *M. tuberculosis* and selected on 50 μg/ml hygromycin B. The transformants were overlaid with Cronex film and the film exposed in complete darkness for a period of time depending on the intensity of the light signal, without the addition of decanal. No light was detected from colonies of *M. smegmatis* transformed with pJS6. The transformations were repeated on several occasions but zero levels of light were detected from the hygromycin resistant colonies. In addition, transformants were also exposed to decanal and measured for the production of light. No light production was seen in the transformants. The *M. smegmatis* electrocompetent cells were also transformed with pSMT1 to check the quality of the cells. pSMT1 (Snewin *et al.*, 1999), an *Escherichia coli-Mycobacterium* shuttle vector, has similar properties to that of pSMT3 but incorporates the *luxAB* genes of *Vibrio harveyi* downstream of the *M. bovis* BCG *hsp*60 gene promoter. Transformants were identified by adding decanal onto the lid of the Petri-dish and exposing Cronex film to the transformants for several minutes. Light was detected from colonies transformed with pSMT1 suggesting that the electrocompetent cells of *M. smegmatis* were acceptable.

M. aurum was also transformed with pJS6 and again no light was detected from these colonies. In addition, pJS5 DNA was also transformed into *M. aurum*, although no light was produced, it did generate more transformants than the pJS6 transformation. Light was detected from colonies of BCG transformed with pJS6 when Cronex film was exposed for approximately 90 minutes in the absence of decanal (Figure 3.5). As a control pSMT1 was transformed into BCG and light was detected when colonies were exposed to decanal and overlaid with Cronex film for approximately 90 minutes (Figure 3.6). Introduction of pJS6 plasmid DNA into *M. tuberculosis* strain H37Rv resulted in colonies that produced light and these could be detected by exposing Cronex film to transformants for approximately 60 minutes in the absence of decanal (Figure 3.7). In addition, high levels of light were detected in *M. tuberculosis* (pJS6) when checked within 20 minutes (Figure 3.8). Overall, bioluminescence was detected in BCG and *M. tuberculosis* but was not detected in *M. smegmatis* or *M. aurum*.

3.2.3. Extraction of pJS6 plasmid DNA from *Mycobacterium smegmatis* mc^2155 and BCG.

To confirm the presence of the plasmid pJS6 in *M. smegmatis* given that no bioluminescence was demonstrated, plasmid DNA was prepared from the recombinant *M. smegmatis* and subsequently the DNA transformed into *E. coli* strain DH5α. Transformants were selected on 200 μg/ml hygromycin B. The recombinant *E. coli* was measured for the production of light by exposing Cronex film to the transformants in complete darkness for several minutes without the addition of decanal (Figure 3.9). High levels of light were detected from transformants indicating that the plasmid had been present in *M. smegmatis*. Plasmid DNA was also prepared from *M. smegmatis* transformed with pSMT1 and subsequently transformed into *E. coli* strain DH5α. Transformants were checked for the production of light by exposing the transformants to decanal and light colonies were seen (Figure 3.10).

Unfortunately, the expression vector, pJS6, was found to be unstable in BCG and *M. tuberculosis*. After several generations, no light was detected in either of the recombinant mycobacteria. BCG that did not express light was found to grow faster than the light producing recombinant BCG. Therefore, plasmid DNA was prepared from BCG (pJS6) and BCG (pSMT1) (used as a control) and transformed into *E. coli* strain DH5α. No light was detected from *E. coli* transformed with pJS6 plasmid DNA

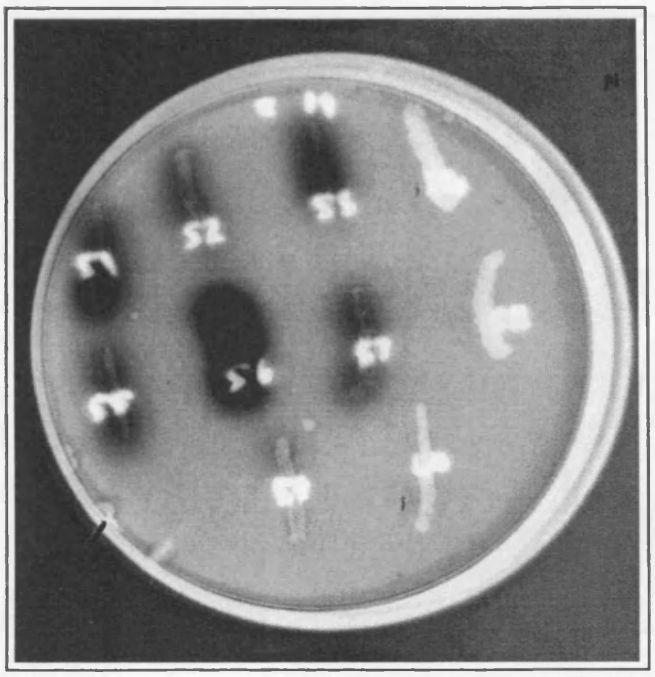


Figure 3.5. Transformation of pJS6 DNA into BCG.

The detection of light from BCG transformed with pJS6 containing the *luxCDABE* genes was done by exposing Cronex film to the transformants in complete darkness for approximately ninety minutes in the absence of decanal. The X-ray film shows the light produced by several colonies containing pJS6 DNA (black streaks). (Photograph of a Petri-dish).

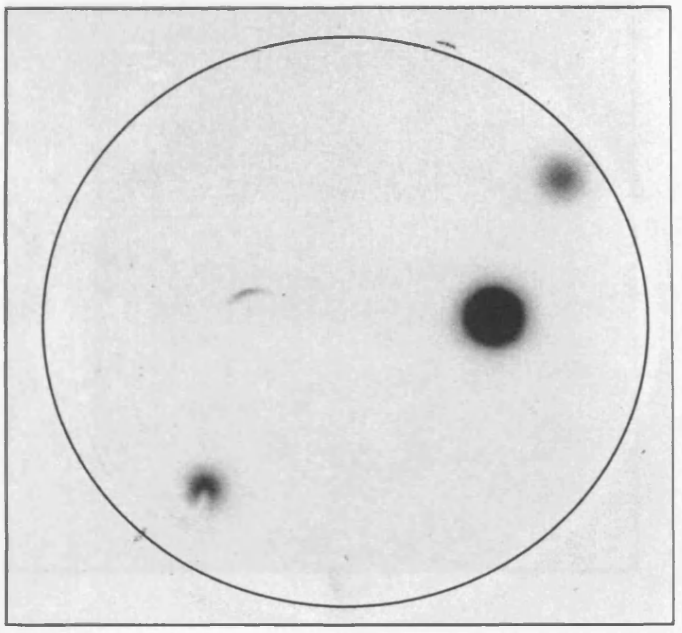


Figure 3.6. Transformation of pSMT1 DNA into BCG.

The detection of light from BCG transformed with pSMT1 containing the *luxAB* genes was done by exposing Cronex film to the transformants in complete darkness for ninety minutes in the presence of decanal. The X-ray film shows colonies that were light (black dots). (Photograph of a Petri-dish).

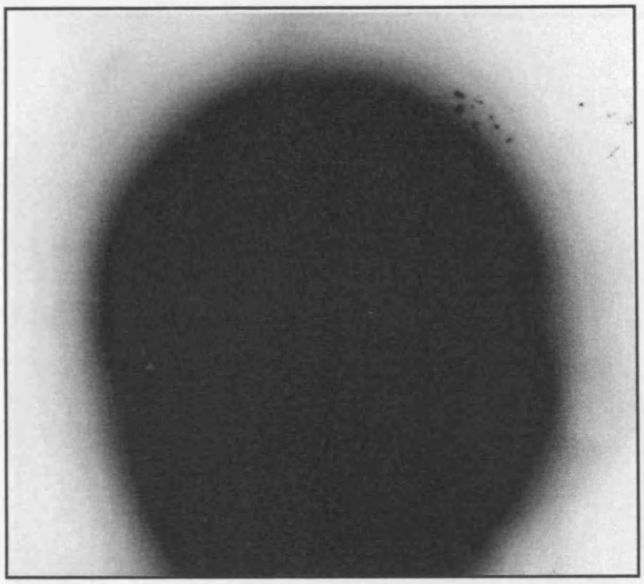


Figure 3.7. Transformation of pJS6 DNA into *Mycobacterium tuberculosis*.
The detection of light from *M. tuberculosis* transformed with pJS6 (original transformants) containing the *luxCDABE* genes was done by exposing Cronex film to the transformants in complete darkness for approximately sixty minutes in the absence of decanal. The X-ray film shows the very high levels of light produced by the numerous colonies transformed with pJS6 on an agar plate (black centre). (Photograph of a Petri-dish).

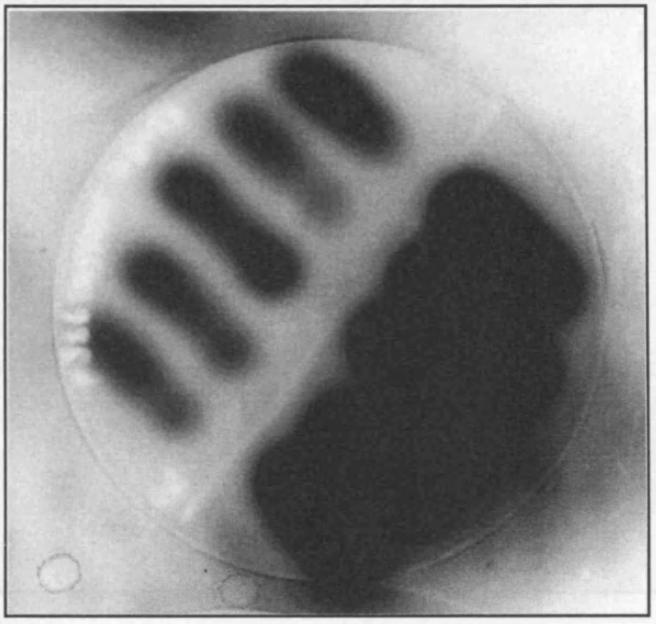


Figure 3.8. Transformation of pJS6 DNA into *Mycobacterium tuberculosis.*
Recombinant *M. tuberculosis* containing pJS6 was streaked out on to fresh medium from the original transformants. The detection of light from *M. tuberculosis* transformed with pJS6 containing the *luxCDABE* genes was done by exposing Cronex film to the transformants in complete darkness for twenty minutes in the absence of decanal. The X-ray film shows two transformants that were light, streaked out several times (black streaks). (Photograph of a Petri-dish).

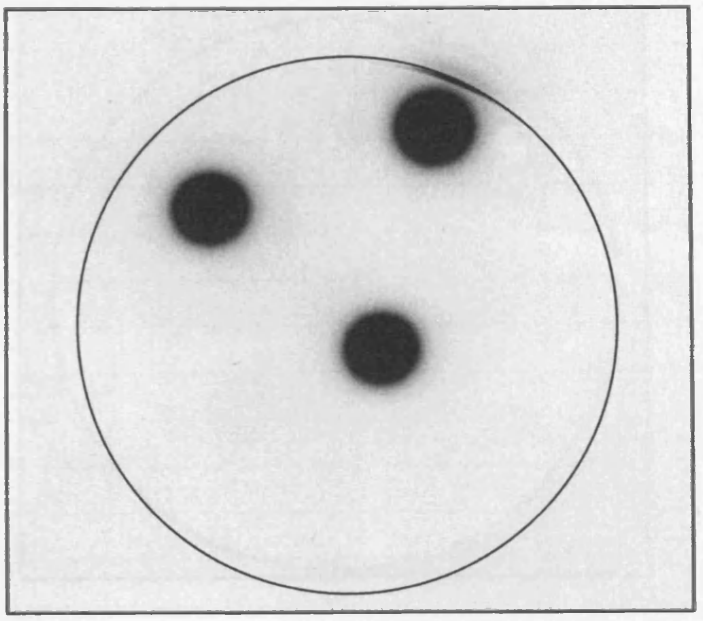


Figure 3.9. Extraction of pJS6 DNA from *Mycobacterium smegmatis*.
To confirm the presence of the plasmid pJS6 in *M. smegmatis*, plasmid DNA was prepared from the recombinant *M. smegmatis* and subsequently the DNA transformed into *E. coli*. The recombinant *E. coli* was measured for the production of light by exposing Cronex film to the transformants in complete darkness for two minutes without the addition of decanal. The X-ray film shows colonies that were light (black dots). (Photograph of a Petri-dish).

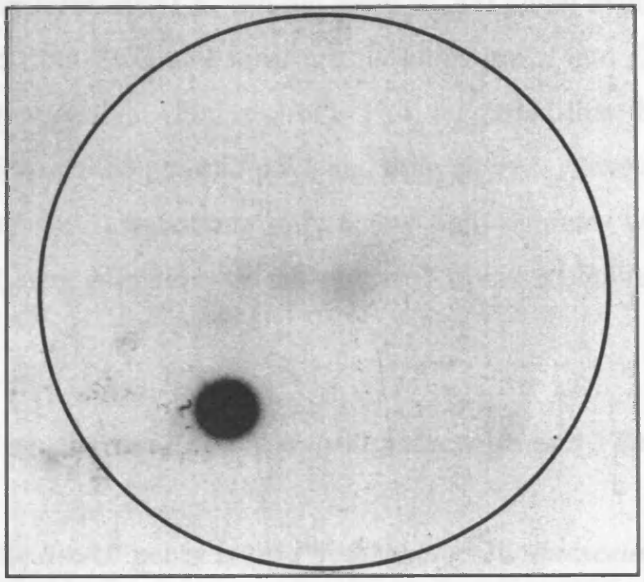


Figure 3.10. Extraction of pSMT1 DNA from *M. smegmatis*.

To confirm the presence of the plasmid pSMT1 in *M. smegmatis*, plasmid DNA was prepared from the recombinant *M. smegmatis* and subsequently the DNA transformed into *E. coli*. The recombinant *E. coli* was measured for the production of light by exposing Cronex film to the transformants in complete darkness for two minutes with the addition of decanal. The X-ray film shows colonies that were light (black and grey dots). (Photograph of a Petri-dish).

extracted from BCG. DNA was prepared from the recombinant *E. coli* but no pJS6 plasmid was detected when run on an agarose gel. Either no plasmid had originally been extracted due to the difficulty in preparing DNA from BCG or the plasmid had been lost. However, transformants of *E. coli* did grow on 200 μg/ml hygromycin. The control pSMT1, extracted from BCG and subsequently transformed into *E. coli* did generate a few colonies that were light (Figure 3.11). This suggested that the methodology had worked and implies that the plasmid pJS6 had been altered. However, the methodology did not work very well considering only a few light colonies were seen, thus, it is possible that sufficient plasmid was not prepared from recombinant BCG containing pJS6.

3.3. The *luxAB* genes from *Photorhabdus luminescens* and *Vibrio harveyi.*

3.3.1. Cloning the *luxAB* genes from *Photorhabdus luminescens.*

The *lux* operon, *luxCDABE* from *Photorhabdus luminescens*, when introduced into *M. smegmatis* did not produce light. To determine whether this occurrence was due to the presence of the complete operon in the cell and thus was resulting in the continual production of aldehyde causing cell toxicity, a vector was constructed using just the *luxAB* genes from *P. luminescens*. The vector would then be introduced into the various strains of mycobacteria and observed for the production of light. The recombinant *Mycobacterium* was generated in collaboration with a colleague (Patel, S. University of Leicester).

The expression vector pSP1 based on *lux* was constructed for use in mycobacteria (Figure 3.12). The *Escherichia coli-Mycobacterium* shuttle vector pSMT3 was used for the basis of construction for the vector, which consisted of an origin of replication for *E. coli* and mycobacteria and a hygromycin resistance gene. The *luxAB* genes of *Photorhabdus luminescens* were obtained from pRDH42 (Haigh, R. University of Leicester). To construct the expression vector, pSP1, the *luxAB* genes from pRDH42, were digested with *Pst* I and *Bam*H I thus resulting in the excision of the *luxAB* fragment of approximately 3 Kb. Subsequently, pSMT3 DNA was digested with *Pst* I and *Bam*H I to linearise the DNA. The 3 Kb *Pst* I/*Bam*H I fragment of *luxAB* was ligated into the *Pst* I/*Bam*H I site of pSMT3. The ligation product was introduced into

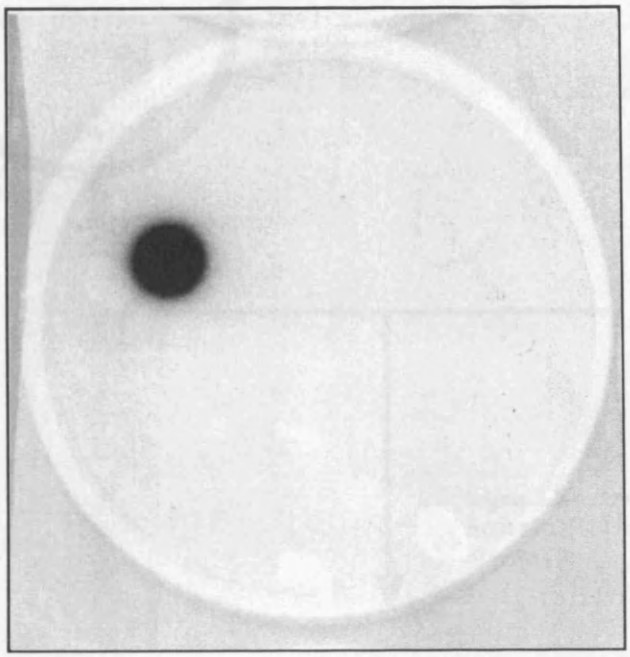


Figure 3.11. Extraction of pSMT1 DNA from BCG.

To confirm the presence of the plasmid pSMT1 in BCG, plasmid DNA was prepared from the recombinant BCG and subsequently the DNA transformed into *E. coli*. The recombinant *E. coli* was measured for the production of light by exposing Cronex film to the transformants in complete darkness for two minutes with the addition of decanal. The X-ray film shows one colony that was light (black dot). (Photograph of a Petri-dish).

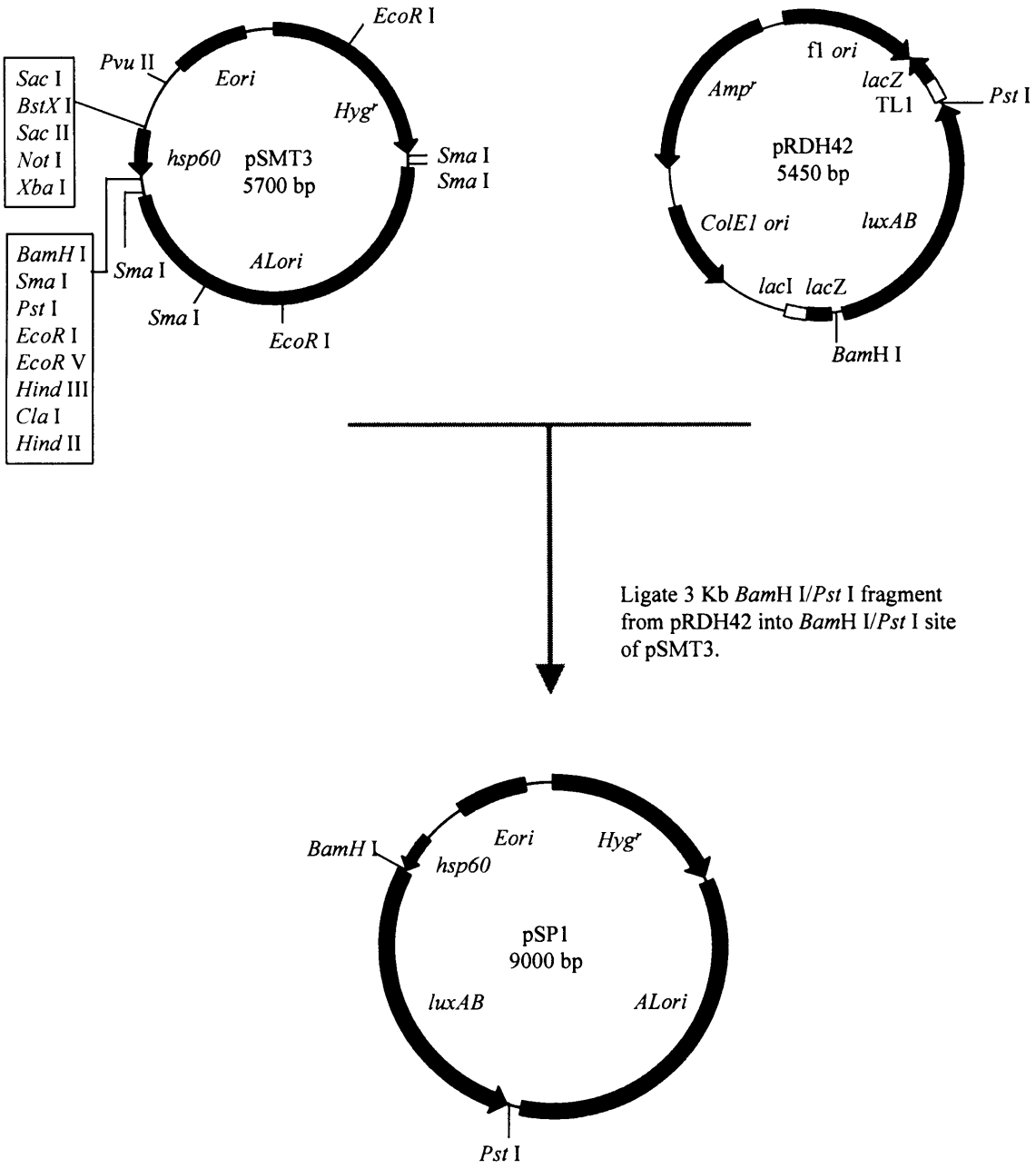


Figure 3.12. Construction of the expression vector, pSP1.

The *luxAB* genes from pRDH42 were digested with *Bam*H I/*Pst* I and ligated into the *Bam*H I/*Pst* I site of pSMT3 to produce pSP1. Abbreviations are mycobacterial origin of replication (*ALori*), *Escherichia coli* origin of replication (*Eori* and *ColE1 ori*), hygromycin resistance (Hyg^r), ampicillin resistance (Amp^r), luciferase genes from *Photorhabdus luminescens* (*luxAB*), terminator sequence (TL1), blue/white selection with β-galactosidase (*lacZ*) and f1 filamentous phage origin of replication (f1 *ori*).

E. coli strain DH5α by electroporation and transformants were selected on 200 μg/ml hygromycin B.

Transformants containing the *luxAB* genes were identified by adding decanal onto the lid of the Petri-dish and exposing Cronex film to the transformants for several minutes depending on the intensity of the light signal. Light colonies were identified and were chosen for further investigation. Plasmid DNA was prepared from the colony. This demonstrated successful cloning of the *luxAB* DNA fragment into pSMT3 and further work was done with the expression vector, pSP1.

3.3.2. Transformation of pSP1 DNA into *Escherichia coli*, *Mycobacterium smegmatis* mc^{2}155 and *Mycobacterium aurum*.

To compare the *luxAB* genes from *Vibrio harveyi* and *Photorhabdus luminescens*, *Escherichia coli*, *Mycobacterium smegmatis* mc^{2}155 and *Mycobacterium aurum* were transformed with plasmid DNA, pSP1, pSMT1 or pPA3. pSMT1 (Snewin *et al.*, 1999) and pPA3 (Andrew and Roberts, 1993) were generated by cloning the *luxAB* genes from *Vibrio harveyi* downstream of the *M. bovis* BCG *hsp*60 gene promoter (Stover *et al.*, 1991) in the *Escherichia coli-Mycobacterium* shuttle vectors pSMT3 and pMV261 respectively. pPA3 transformants were selected on 20 μg/ml kanamycin and pSP1 and pSMT1 were selected on either 50 μg/ml or 200 μg/ml hygromycin B.

Transformed plasmids pSP1, pSMT1 and pPA3 were measured for the production of light by adding decanal onto the lid of the Petri-dish and exposing Cronex film to the transformants in complete darkness for 10 to 15 minutes. Results are shown in Table 3.1. Light was observed from recombinant *E. coli* transformed with pSP1, pSMT1 or pPA3. No light was detected from *M. smegmatis* transformed with pSP1. However, light was detected in *M. smegmatis* (pSMT1) and *M. smegmatis* (pPA3), thus demonstrating that there are differences in the expression of the *luxAB* genes from *P. luminescens* and *V. harveyi* in *M. smegmatis*. Light was detected from *M. aurum* when transformed with pSP1 (Figure 3.13.), pSMT1 or pPA3, thus, indicating that the *luxAB* genes from *P. luminescens* and *V. harveyi* do function in *M. aurum*. After several generations pSP1 was found to be stable in *M. aurum*.

Table 3.1. Light production from recombinant bacteria expressing *lux* genes.

Vector	Strain	*lux* genes	*E. coli*	*M. smegmatis*	*M. aurum*
pSP1	Pl (Swift *et al.*, 1995)	AB	√	X	√
pJS6	Pl (Swift *et al.*, 1995)	CDABE	√	X	X
pPA3	Vh (Hill *et al.*, 1991)	AB	√	√	√
pSMT1	Vh (Snewin *et al.*, 1999)	AB	√	√	√

Pl = *Photorhabdus luminescens*

Vh = *Vibrio harveyi*

√ = Light

X = Dark

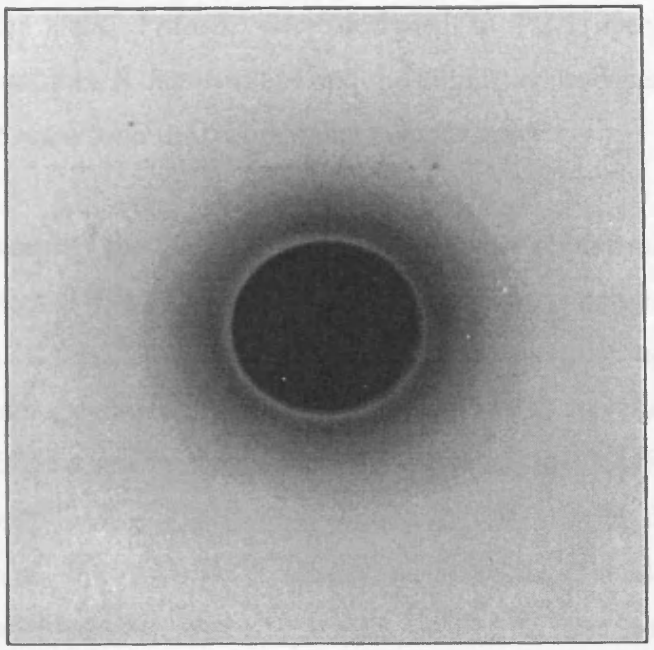


Figure 3.13. Transformation of pSP1 DNA into *Mycobacterium aurum.*
The detection of light from *M. aurum* transformed with pSP1 containing the *luxAB* genes was done by exposing Cronex film to the transformant in complete darkness for five minutes in the presence of decanal. The X-ray film shows a liquid culture of a recombinant *M. aurum* that was light (black circle). (Photograph of a Petri-dish).

3.4. Transcription of the *luxCDABE* operon in mycobacteria.

Northern hybridisation was done to analyse gene transcription of the *luxCDABE* genes in *M. smegmatis* and BCG to explain why bioluminescence was not detected or why cells had become dark. Primers were designed to PCR specific regions of the *luxCDABE* operon from *P. luminescens* and the amplified products were used to probe against RNA extracted from the recombinant mycobacteria.

3.4.1. PCR to amplify the *luxC*, *luxD* and *luxAB* gene sequence.

Primers were designed to amplify regions of the *luxCDABE* operon. Primers LuxC-HB1 (forward) and LuxC-HB2 (reverse) were designed to amplify the *luxC* gene (Meighen *et al.*, 1992). Primers LuxD-HB1 (forward) and LuxD-HB2 (reverse) were designed to amplify the *luxD* gene and primers LuxA-HB (forward) and LuxB-HB (reverse) were designed to amplify the *luxAB* gene (Meighen *et al.*, 1992) using the polymerase chain reaction (Saiki *et al.*, 1988). The PCR of *luxC*, *luxD* and *luxAB* was done as described in the Materials and Methods (section 2.5. and Table 2.3). The *luxC*, *luxD* and *luxAB* genes were amplified from the plasmid pJS6. The PCR products were visualised at approximately 1450 bp, 1000 bp and 2000 bp for the *luxC*, *luxD* and *luxAB* genes respectively, on a 0.7% (w/v) agarose gel. These bands were excised from the agarose gel and DNA was isolated using the Sephaglas Bandprep Kit. These DNA fragments were used as probes against RNA extracted from the recombinant mycobacteria.

3.4.2. Transcription of the *lux* operon in mycobacteria.

RNA was extracted from cultures of *M. smegmatis* (pJS6), *E. coli* (pJS6) and *E. coli* as described in the Materials and Methods (sections 2.14.1., and 2.14.2). The RNA was separated on an agarose gel by electrophoresis and transferred to a nylon membrane by Northern transfer (sections 2.14.3 and 2.14.4). The amplified PCR fragments *luxC*, *luxD* and *luxAB* were labelled with [α-^{32}P]dCTP and used as hybridisation probes (sections 2.12.2). RNA extracted from *E. coli* containing no plasmid was used as a negative control and the RNA extracted from *E. coli* (pJS6) was used as a positive control. Radiolabelled *luxC* and *luxAB* probes were hybridised against RNA. A *luxD* probe was not used in this experiment. The radiolabelled *luxC* and *luxAB* probes did not hybridise to the RNA extracted from *M. smegmatis* (pJS6) (Figure 3.14). Both probes did hybridise to *E. coli* (pJS6). More than one band was observed suggesting that some plasmid DNA may have been present in the RNA samples during extraction.

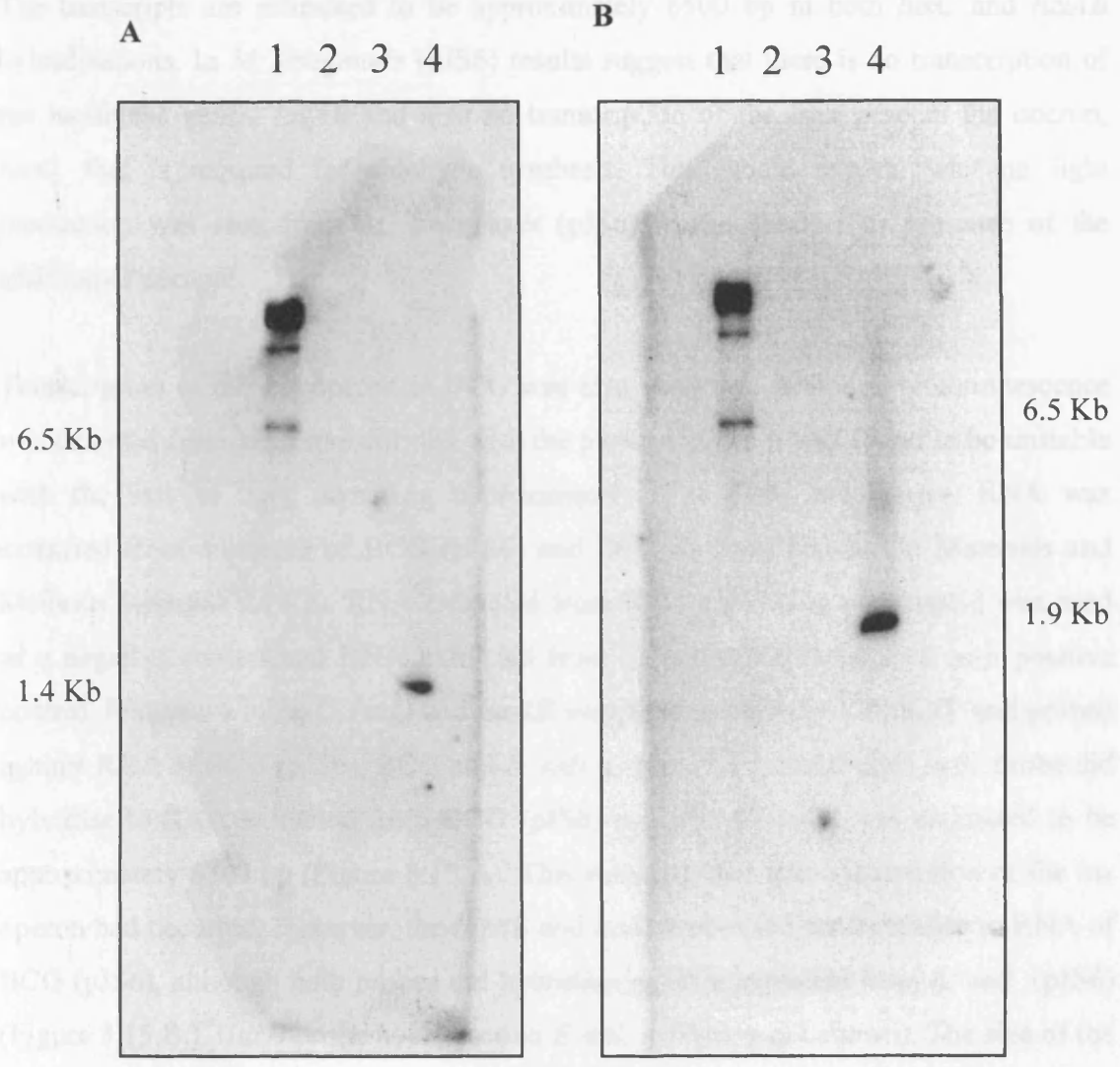


Figure 3.14. Transcription of the *lux* operon in *Mycobacterium smegmatis*.
RNA was extracted from cultures of *M. smegmatis* (pJS6), *E. coli* (pJS6) and *E. coli*. The RNA was separated on an agarose gel by electrophoresis and transferred to a nylon membrane by Northern transfer. The amplified PCR fragments *luxC* and *luxAB* were labelled with [α-^{32}P]dCTP and used as hybridisation probes. Photograph A, lane 2, radiolabelled *luxC* probe hybridised against *M. smegmatis* (pJS6) RNA. Photograph B, lane 2, radiolabelled *luxAB* probe hybridised against *M. smegmatis* (pJS6) RNA. RNA extracted from *E. coli* (pJS6) was used as a positive control (Photographs A and B, lanes 1). RNA extracted from *E. coli* containing no plasmid was used as a negative control (Photographs A and B, lanes 3). Photographs A and B, lanes 4, *luxC* and *luxAB*, PCR product respectively used as a positive control.

The transcripts are estimated to be approximately 6500 bp in both *luxC* and *luxAB* hybridisations. In *M. smegmatis* (pJS6) results suggest that there is no transcription of the luciferase genes, *luxAB* and also no transcription of the first gene in the operon, *luxC*, that is required for aldehyde synthesis. This would explain why no light production was seen from *M. smegmatis* (pJS6) in the absence or presence of the addition of decanal.

Transcription of the *lux* operon in BCG was also analysed. Although bioluminescence was detected from cells transformed with the plasmid pJS6, it was found to be unstable with the loss of light occurring approximately after three subcultures. RNA was extracted from a culture of BCG (pJS6) and BCG as described in the Materials and Methods (sections 2.14.2). RNA extracted from BCG containing no plasmid was used as a negative control and RNA extracted from *E. coli* (pJS6) was used as a positive control. Fragments of *luxC*, *luxD* and *luxAB* were labelled with [α-^{32}P]dCTP and probed against RNA of BCG (pJS6), BCG and *E. coli* (pJS6). The radiolabelled *luxC* probe did hybridise to RNA extracted from BCG (pJS6) and the transcript was estimated to be approximately 6500 bp (Figure 3.15.A). This demonstrated that transcription of the *lux* operon had occurred. However, the *luxAB* and *luxD* probes did not hybridise to RNA of BCG (pJS6), although both probes did hybridise to RNA extracted from *E. coli* (pJS6) (Figure 3.15.B.), (*luxD* probe hybridised to *E. coli* (pJS6) is not shown). The size of the transcripts was estimated to be over 6500 bp. Thus no clear interpretation of the results can be made because although the 6.5 Kb transcript seen with *luxC* probe was consistent with the complete transcript being present, the results with the *luxAB* and *luxD* probes was not consistent with this conclusion, since the *lux* operon is transcribed in the order *lux-C-D-A-B-E*, no interpretation can be made about the transcription of the *lux* operon to explain why light production was not stable in BCG (pJS6).

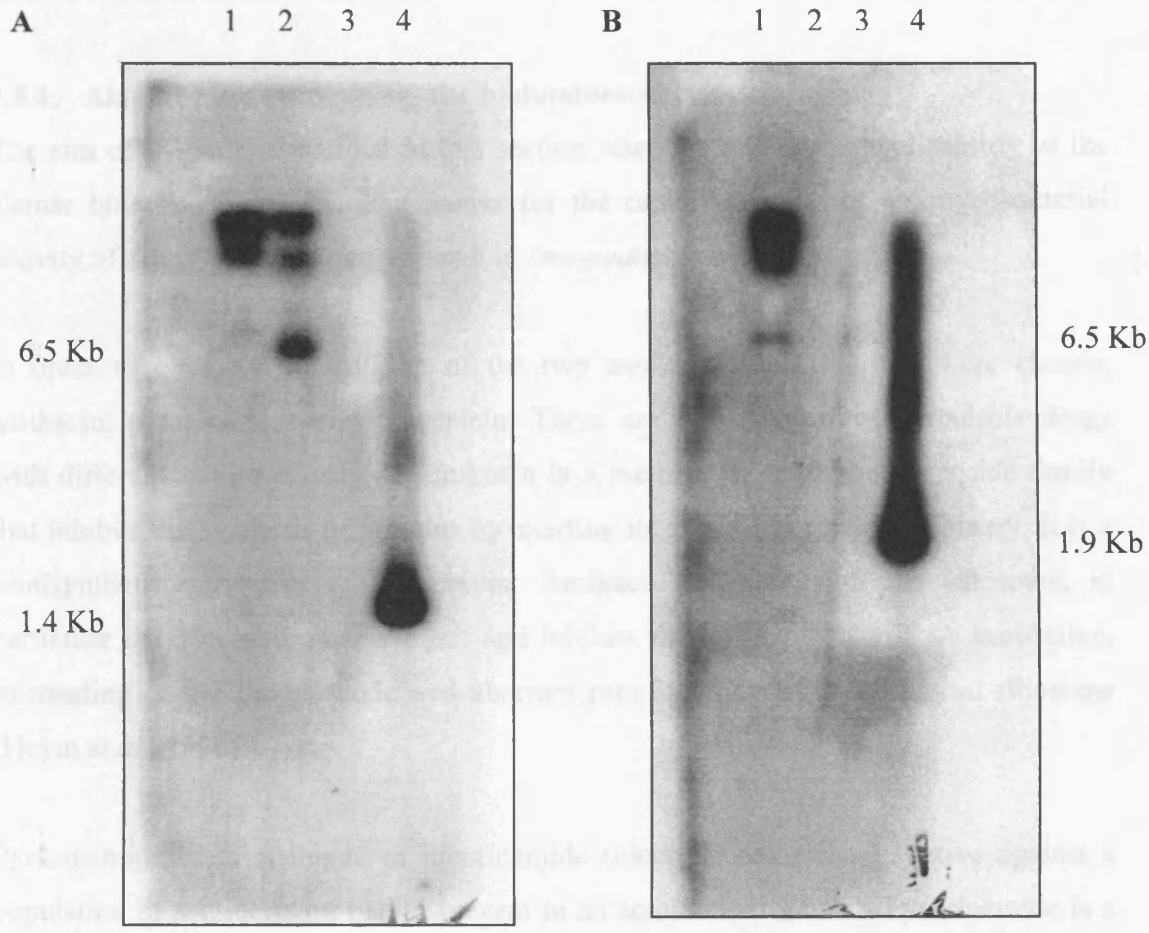


Figure 3.15. Transcription of the *lux* operon in BCG.

RNA was extracted from cultures of BCG (pJS6), *E. coli* (pJS6) and *E. coli*. The RNA was separated on an agarose gel by electrophoresis and transferred to a nylon membrane by Northern transfer. The amplified PCR fragments *luxC* and *luxAB* were labelled with [α-^{32}P]dCTP and used as hybridisation probes. Photograph A, lane 2, radiolabelled *luxC* probe hybridised against BCG (pJS6) RNA. Photograph B, lane 2, radiolabelled *luxAB* probe hybridised against BCG (pJS6) RNA. RNA extracted from *E. coli* (pJS6) was used as a positive control (Photographs A and B, lanes 1). RNA extracted from *E. coli* containing no plasmid was used as a negative control (Photographs A and B, lanes 3). Photographs A and B, lanes 4, *luxC* and *luxAB*, PCR product respectively used as a positive control.

3.5. Bioluminescence and its uses.

3.5.1. Alamar blue assay verses the bioluminescence assay.

The aim of the work described in this section was to compare the applicability of the alamar blue and bioluminescent assays for the rapid screening of antimycobacterial activity of drugs against *M. aurum* and *M. smegmatis*.

In order to compare the validity of the two assays, three antibiotics were chosen, amikacin, pyrazinamide and rifampicin. These are important anti-tuberculosis drugs with different modes of action. Amikacin is a member of the aminoglycoside family that inhibits the synthesis of proteins by exerting its effect on protein machinery. It is a semisynthetic derivative of kanamycin. Amikacin interacts with the ribosome, in particular the 30S ribosomal subunit and inhibits the initiation of mRNA translation, misreading of the genetic code and aberrant proof-reading by the bacterial ribosome (Heym *et al.*, 1996).

Pyrazinamide is an analogue of nicotinamide (nicotinic acid) and is active against a population of semidormant bacilli present in an acidic environment. Pyrazinamide is a pro-drug that is activated by the bacterial enzyme, nicotinamidase-pyrazinamidase (Pzase), into pyrazinoic acid (POA), the active form of the drug. Pzase is encoded by the *pncA* gene (Zhang *et al.*, 1999). Under acidic conditions, pyrazinamide is transported into the cell and is converted into pyrazinoic acid. Accumulation of pyrazinoic acid into the cell results in susceptibility of *M. tuberculosis* to pyrazinamide (Zhang *et al.*, 1999).

Rifampicin is an inhibitor of bacterial DNA-dependent RNA polymerase. RNA polymerase is a complex oligomer that is composed of four different subunits (α, β, β' and σ). The four subunits are encoded by the genes *rpoA*, *rpoB*, *rpoC* and *rpoD*. Rifampicin inhibits RNA polymerase by covalently binding to the β-subunit, which is involved in chain initiation and elongation, and thus obstructs transcription (Heym *et al.*, 1996).

Mycobacterium smegmatis (pSMT1) and *Mycobacterium aurum* (pPA3) were used for both assays. Results for the bioluminescent assay have been presented as minimum light

inhibition (MLI). MLI is defined as the lowest concentration of the drug that results in complete inhibition of light production. Results for the alamar blue assay have been presented as minimum reduction change (MRC). MRC is defined as the lowest concentration of the drug that results in maximal inhibition of alamar blue reduction.

The MLI and MRC for *M. smegmatis* and *M. aurum* were determined by treatment with amikacin, pyrazinamide or rifampicin with incubation for 24 hours for the bioluminescent assay and for the alamar blue assay. Amikacin, pyrazinamide and rifampicin were used at concentrations ranging from 250 to 0.01 μg/ml. After incubation for 24 hours, cultures were measured for bioluminescence in a luminoskan luminometer with the addition of decanal. For the alamar blue assay, after 24 hours incubation alamar blue solution was added to each well. The plates were incubated for an additional 24 hours and the results recorded. Antimycobacterial activity was assessed visually and fluorometrically. Colony-forming units (CFUs) were also determined for *M. smegmatis*.

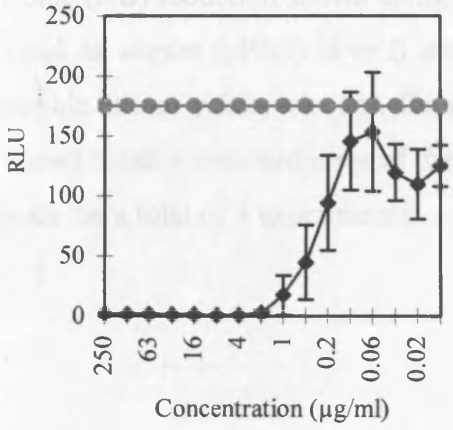
Overall, for *M. smegmatis* the results suggest agreement between the bioluminescence and the alamar blue assays. The findings for two assays with *M. smegmatis* are shown in (Figure 3.16). By bioluminescence, the MLI for amikacin and rifampicin against *M. smegmatis* were 2 μg/ml and 16 μg/ml respectively. No MLI for pyrazinamide was found (>250 μg/ml) (Figure 3.16.a-c). When recorded visually, the alamar blue assay gave essentially the same value for the MRC. For amikacin and rifampicin the MRC against *M. smegmatis* were 1 μg/ml and 16 μg/ml, respectively. No MRC for pyrazinamide was found (Figure 3.16.d-f). The alamar blue assay also was assessed fluorometrically with a view to improving automation. However, as can be seen from Figure 3.17.a-c., the fluorescence did not fall to the baseline with any of the drugs, regardless of concentration, and in most cases was not less than the drug-free control.

With *M. aurum* both assay methods gave similar values for rifampicin and pyrazinamide but not for amikacin. The values of the MLI for amikacin and rifampicin were 1 μg/ml and 0.5 - 1 μg/ml respectively using the bioluminescent assay.

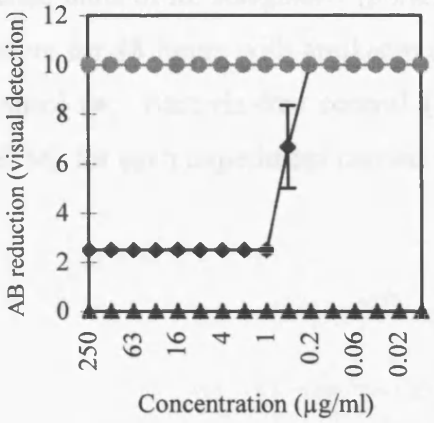
Figure 3.16. Bioluminescent assay and alamar blue assay of *M. smegmatis* (pSMT1).

Bioluminescent assay of *M. smegmatis* (pSMT1) (a to c) after incubation for 24 hours and alamar blue assay of *M. smegmatis* (pSMT1) (d to f) after incubation for 48 hours with amikacin (♦), pyrazinamide (■) or rifampicin (□). Bacteria-free control (▲). Drug free control (●). Graph shows mean ± standard error of the mean (SEM) for each experiment carried out in triplicate for a total of 3 experiments. (RLU-relative light units). For the alamar blue assay pink wells scored 10, blue wells scored of 0.

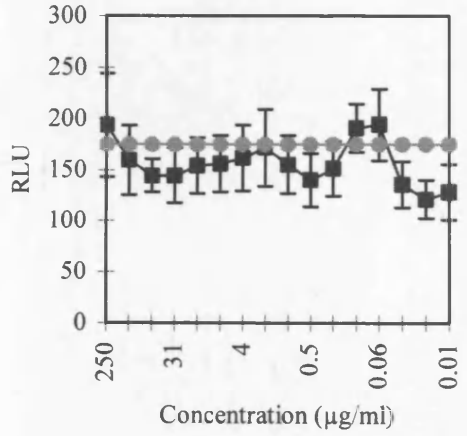
Amikacin (a)



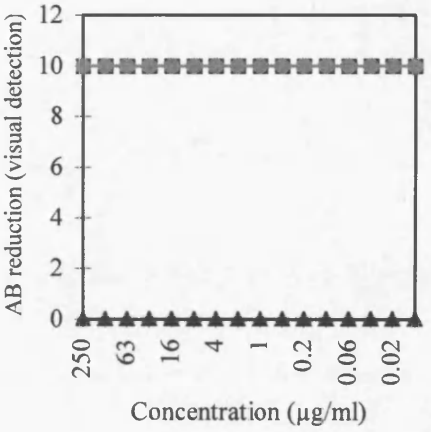
Amikacin (d)



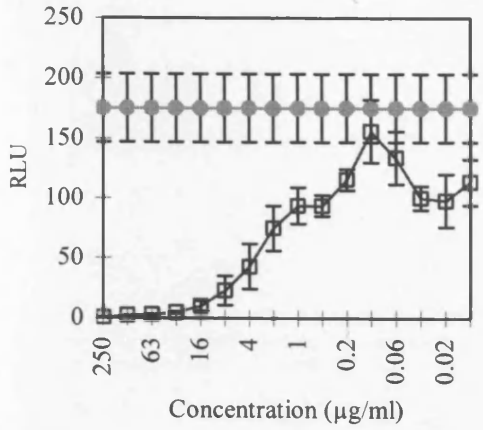
Pyrazinamide (b)



Pyrazinamide (e)



Rifampicin (c)



Rifampicin (f)

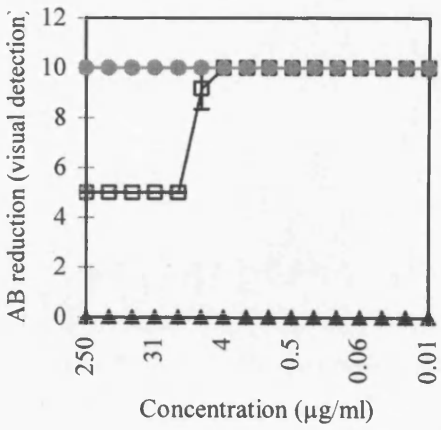
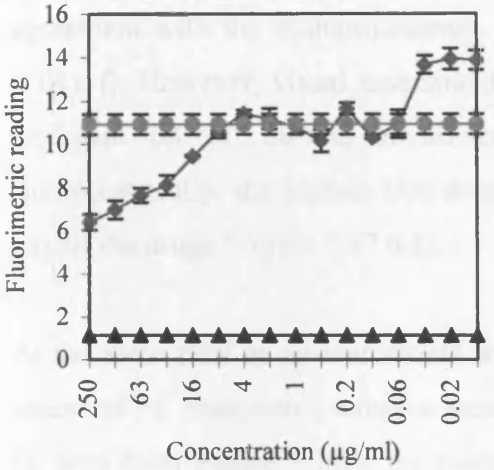


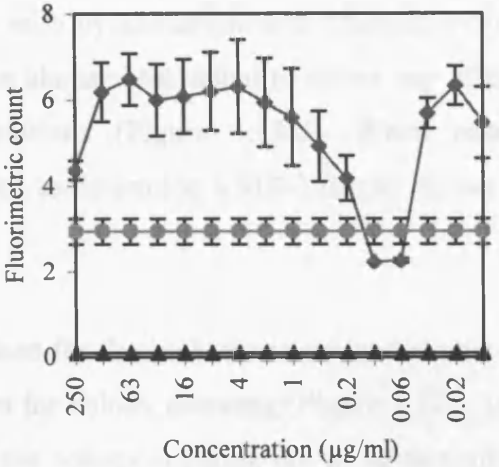
Figure 3.17. Alamar blue assay.

Alamar blue (AB) reduction shown using fluorescence units of *M. smegmatis* (pSMT1) (a to c) and *M. aurum* (pPA3) (d to f) after incubation for 48 hours with amikacin (♦), pyrazinamide (■) or rifampicin (□). Drug free control (●). Bacteria-free control (▲). Graph shows mean ± standard error of the mean (SEM) for each experiment carried out in triplicate for a total of 3 experiments.

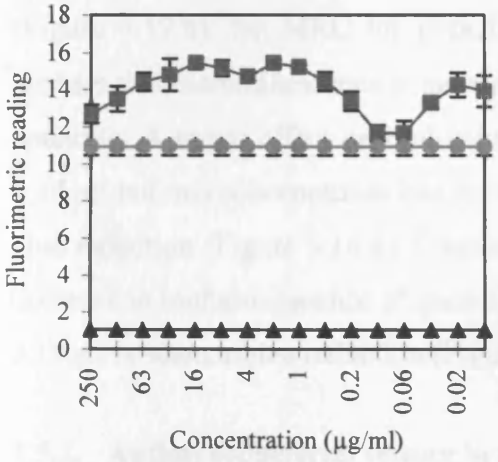
Amikacin (a)



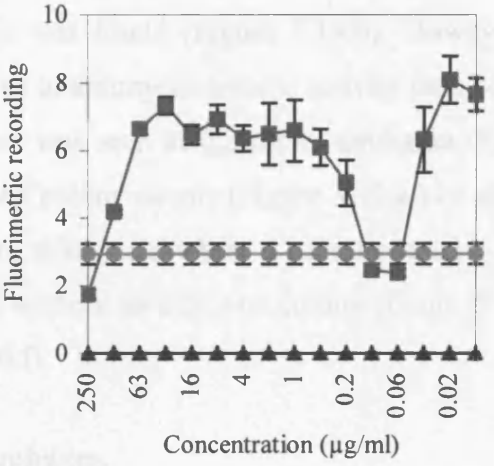
Amikacin (d)



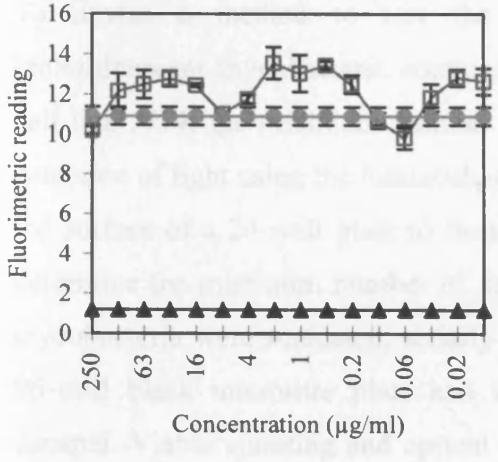
Pyrazinamide (b)



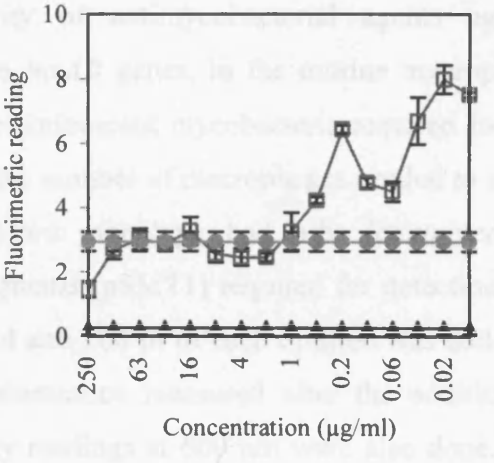
Pyrazinamide (e)



Rifampicin (c)



Rifampicin (f)



No MLI for pyrazinamide was found (Figure 3.18.a-c). When assessed visually, with the alamar blue assay the MRC for pyrazinamide and rifampicin were in good agreement with the bioluminescence assay with pyrazinamide and rifampicin (Figure 3.18.e-f). However, visual assessment of the alamar blue failed to detect any effect of amikacin on *M. aurum*, at all concentrations (Figure 3.18.d). When assessed fluorometrically, the alamar blue assay failed to determine a MRC for *M. aurum* with any of the drugs (Figure 3.17.d-f).

At the same time as measurements were taken for the bioluminescent and alamar blue assays of *M. smegmatis*, samples were taken for colony counting (Figure 3.19). As can be seen from Figure 3.19.b, the results of the colony counting are in agreement with those of the alamar blue assay (Figure 3.16.d-f). For amikacin, the colony counts suggest a MRC between 0.5 and 4 μg/ml and for rifampicin between 4 and 31 μg/ml (Figure 3.19.b). No MRC for pyrazinamide was found (Figure 3.19.b). However, it appears that bioluminescence is more sensitive to antimycobacterial activity than colony counting. A major effect on bioluminescence was seen at 0.2 μg/ml amikacin (Figure 3.16.a.) but this concentration has no effect on colony counts (Figure 3.19.a.) or alamar blue reduction (Figure 3.16.d). Likewise with rifampicin where 4 μg/ml caused a large decrease in bioluminescence (Figure 3.16.c.) without an effect on colony counts (Figure 3.19.a.) or alamar blue reduction (Figure 3.16.f).

3.5.2. Antimycobacterial testing in macrophages.

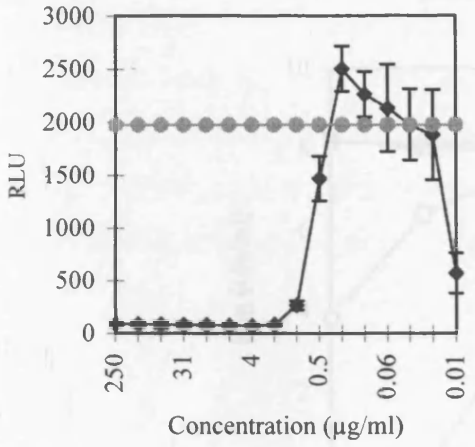
3.5.2.1. Detection of mycobacteria.

To devise a method to test the activity of antimycobacterial agents against bioluminescent mycobacteria containing the *luxAB* genes, in the murine macrophage cell line J774, the minimum number of bioluminescent mycobacteria required for the detection of light using the luminoskan and the number of macrophages needed to cover the surface of a 24-well plate to form a uniform monolayer had to be determined. To determine the minimum number of *M. smegmatis* (pSMT1) required for detection, the mycobacteria were sonicated, serially diluted and 100 μl of each dilution was added to 96-well black microtitre plate and bioluminescence measured after the addition of decanal. Viable counting and optical density readings at 600 nm were also done. The experiments were done using two different cultures (Figure 3.20). The minimum

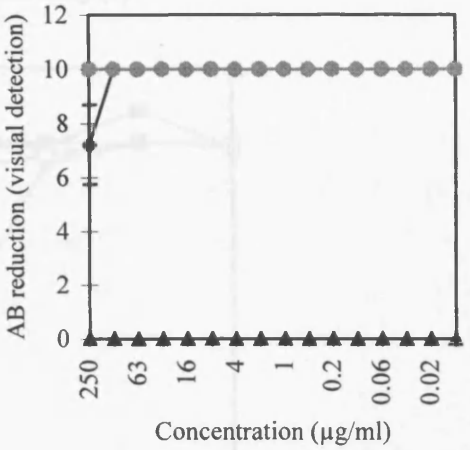
Figure 3.18. Bioluminescent assay and alamar blue assay of *M. aurum* (pPA3).

Bioluminescent assay of *M. aurum* (pPA3) (a to c) after incubation for 24 hours and alamar blue assay of *M. aurum* (pPA3) (d to f) after incubation for 48 hours with amikacin (♦), pyrazinamide (■) or rifampicin (□). Drug free control (●). Bacteria-free control (▲). Graph shows mean ± standard error of the mean (SEM) for each experiment carried out in triplicate for a total of 3 experiments. (RLU-relative light units). For the alamar blue assay pink wells scored 10, blue wells scored of 0.

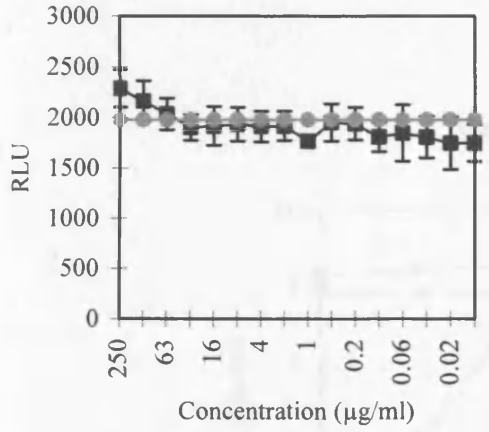
Amikacin (a)



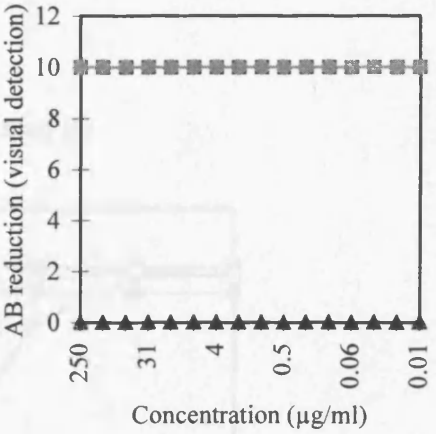
Amikacin (d)



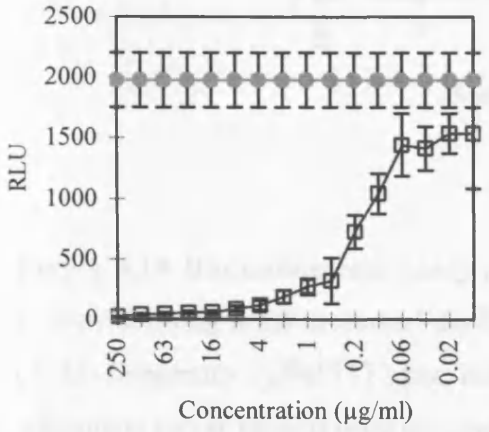
Pyrazinamide (b)



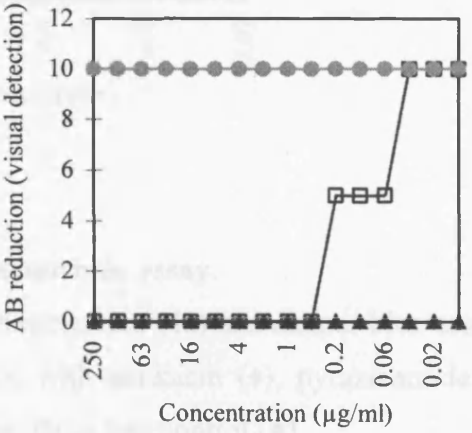
Pyrazinamide (e)



Rifampicin (c)



Rifampicin (f)



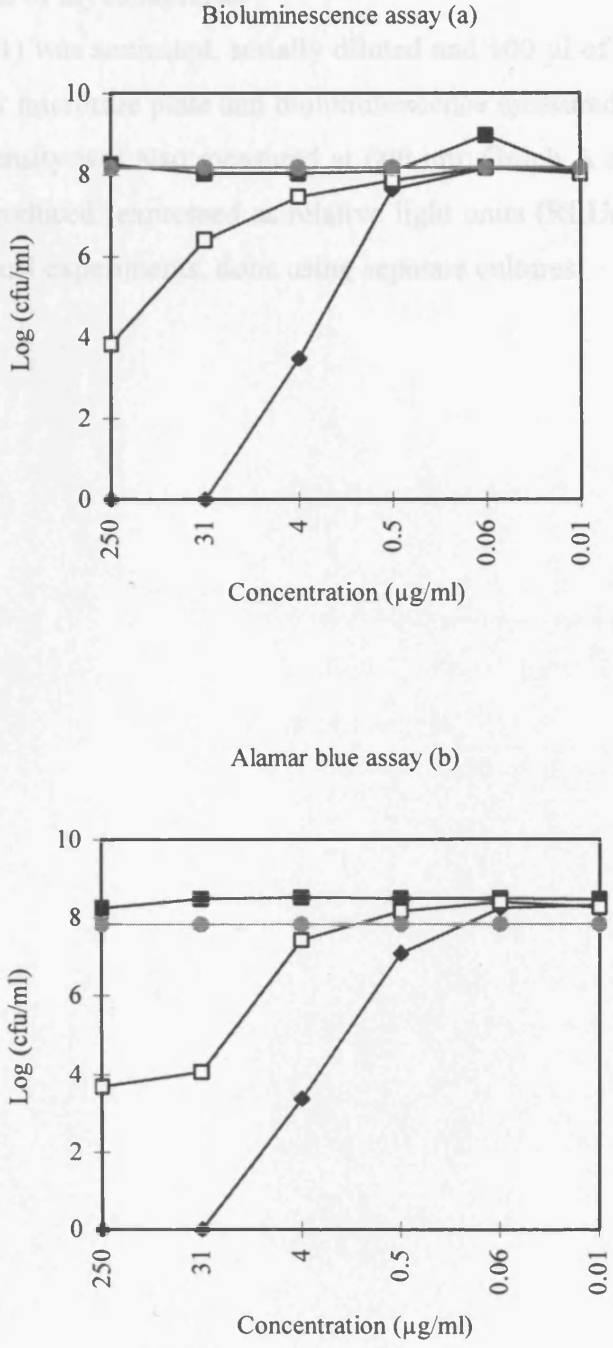


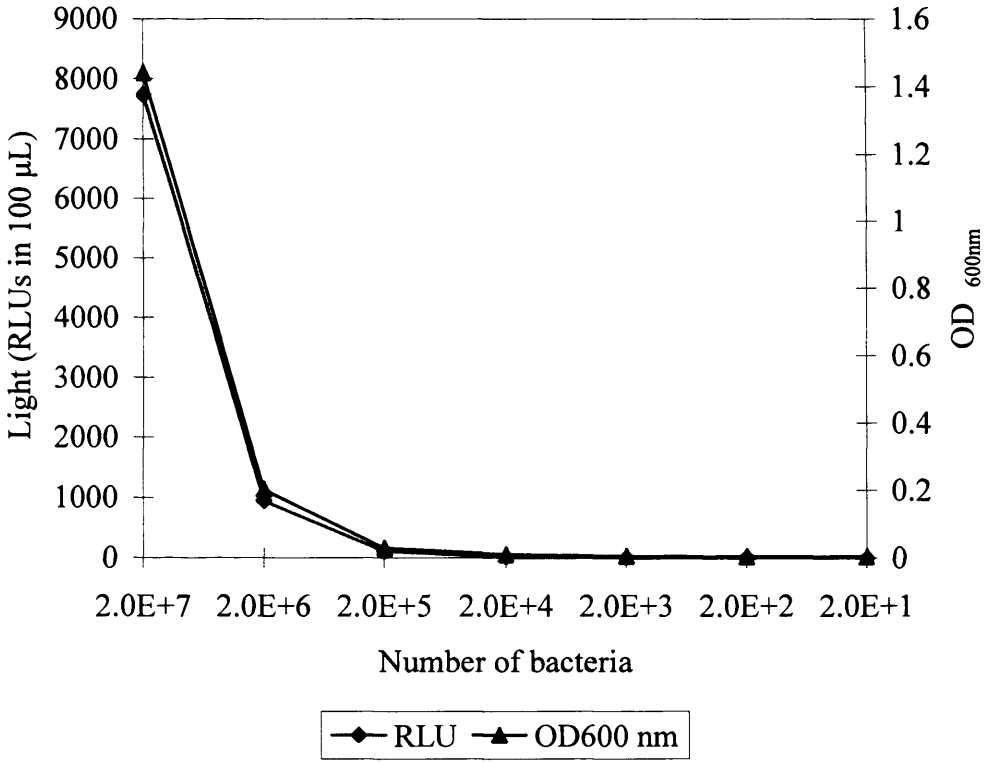
Figure 3.19. Bioluminescent assay and alamar blue assay.

Colony forming units measured during bioluminescent (4a) and alamar blue assay (4b) of *M. smegmatis* (pSMT1) after incubation with amikacin (♦), pyrazinamide (■) or rifampicin (□) at various drug concentrations. Drug free control (●).

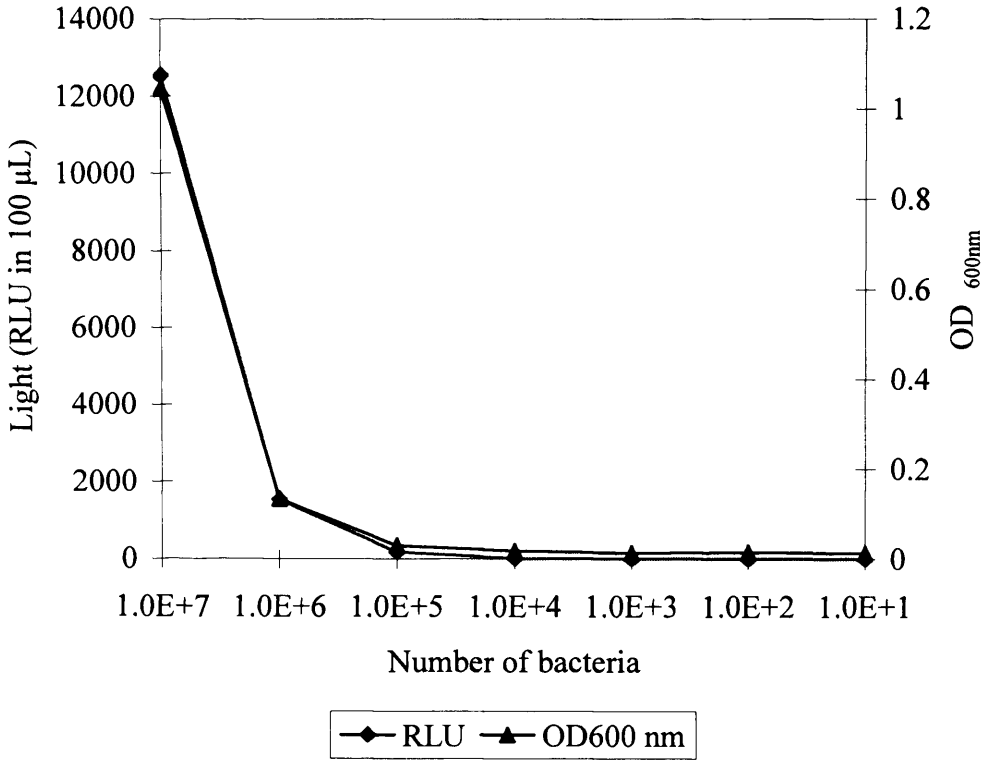
Figure 3.20. Detection of mycobacteria.

M. smegmatis (pSMT1) was sonicated, serially diluted and 100 µl of each dilution was added to 96-well black microtitre plate and bioluminescence measured after the addition of decanal. Optical density was also measured at 600 nm. Graph A and graph B show the amount of light produced (expressed as relative light units (RLUs)) and the optical density of two individual experiments, done using separate cultures.

Culture 1.



Culture 2.



number of bioluminescent mycobacteria required for detection in the luminoskan was 1 x 10^5 bacteria in 100 µl, which gave 105 relative light units.

3.5.2.2. Preparation of the monolayer.

To determine the number of macrophages needed to cover the surface of a 24-well plate as a uniform monolayer, macrophages were prepared, counted and then added to the wells at different concentrations. The volume of medium in each of these wells was also varied between 200 µl to 500 µl. From this initial experiment, a further experiment was done to increase the number of macrophages in a volume of 350 µl (Table 3.2). A volume greater than this resulted in many of the cells attaching to the sides of the well, rather than to the bottom surface. After 24 hours incubation, cells were washed and 350 µl or 300 µl of fresh medium added to each well. The monolayer was observed and the results are shown in Table 3.2. 3 x 10^7 macrophages in a volume of 350 µl covered the surface of the well completely and this concentration was used for subsequent experiments. 350 µl volumes were used for incubation of the cells because this volume covered the cells adequately without drying out over the duration of the experiment.

3.5.2.3. Removal of extracellular mycobacteria.

The aim of this experiment was to determine how effective the procedure used to wash the monolayer was in removing extracellular mycobacteria. Macrophages were infected with *M. aurum* (pPA3) and after incubation for four hours the supernatant was removed and bioluminescence measured from *M. aurum* (pPA3). The viability of the cultures was also estimated after each wash by determining colony-forming units. Monolayers were washed four times so as to remove the majority of the extracellular mycobacteria before the addition of antibiotic to the macrophages. These extracellular mycobacteria were either free in the medium or were contained within macrophages that had detached from the surface of the monolayer. The results demonstrate that after washing the monolayer, the number of relative light units decrease and also washes three and four were the same suggesting that the mycobacteria in the supernatant had been effectively removed (Figure 3.21). Furthermore, there was approximately a one-log drop in the number of colony-forming units after four washes, thus, more than 90% of the extracellular mycobacteria are removed from the monolayer indicating that the wash procedure is effective (Figure 3.21).

Table 3.2. Preparation of the monolayer.

Volume	350 µl	300 µl
Macrophage		
5×10^6	-	-
8×10^6	+	+
1×10^7	++	++
3×10^7	+++	+++

- = Cells very sparse.

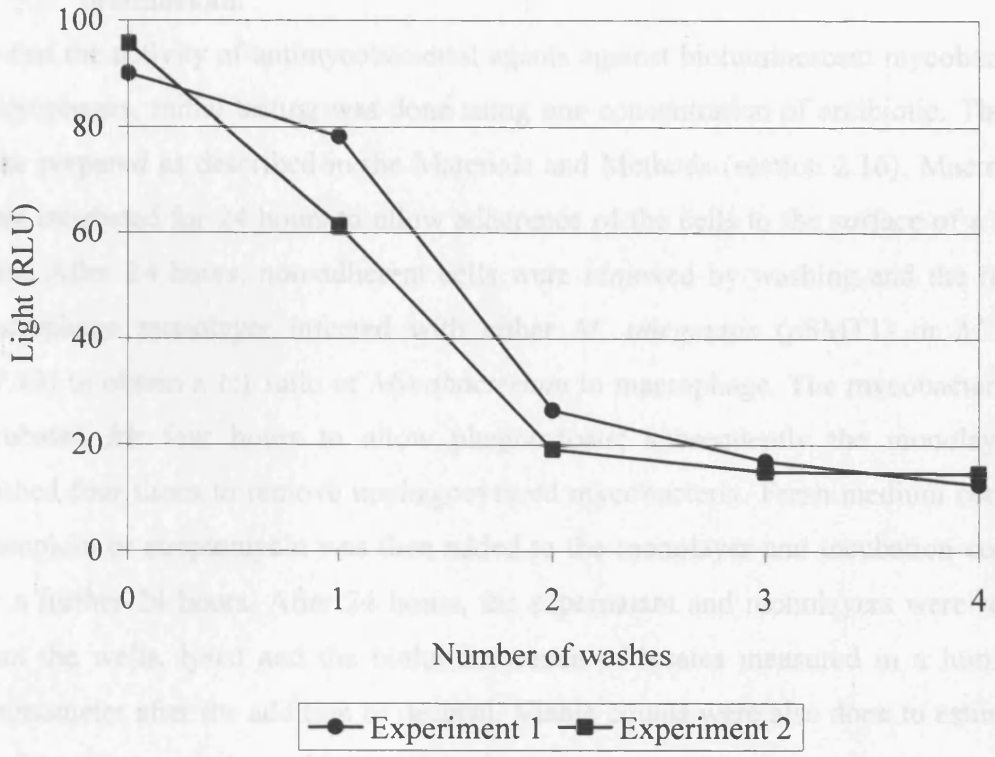
+ = Semi-confluent in some areas of the well.

++ = Confluent in some areas but with a few sparse areas.

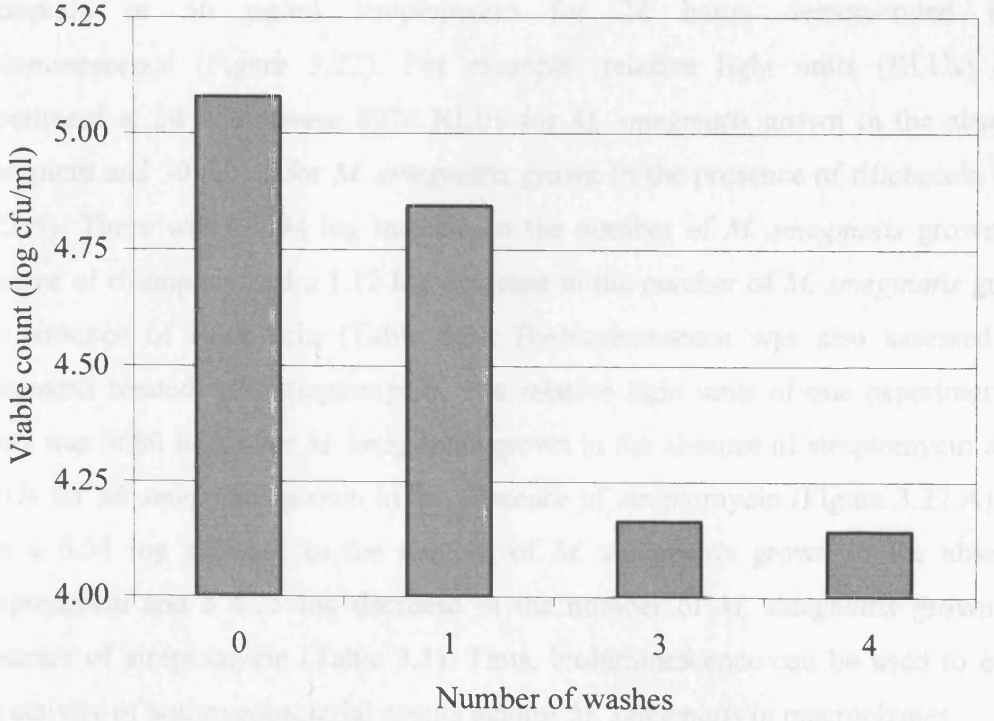
+++ = Confluent in all areas of the well.

Figure 3.21. Experiments to observe the removal of extracellular mycobacteria.
Macrophages were infected with *M. aurum* (pPA3) and after incubation for four hours the supernatant was removed and the light measured. Graph A shows the amount of light produced by *M. aurum* associated with the supernatant after each wash for a total of four washes. Graph B shows the log colony forming units/ml for each wash.

A


100
80
60
40
20
0
Light (RLU)
0
1
2
3
4
Number of washes
Experiment 1
Experiment 2

B


5.25
5.00
4.75
4.50
4.25
4.00
Viable count (log cfu/ml)
0
1
3
4
Number of washes

3.5.2.4. Testing antimycobacterial activity from the expression of *lux* using a luminoskan.

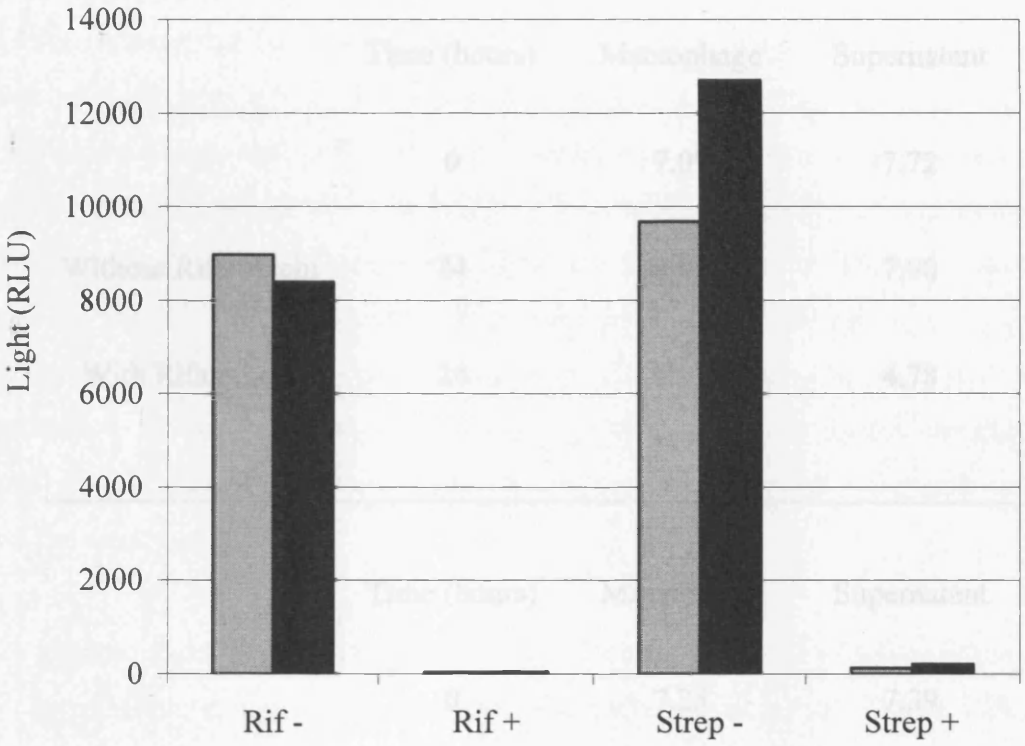
To test the activity of antimycobacterial agents against bioluminescent mycobacteria in macrophages, initial testing was done using one concentration of antibiotic. The J774s were prepared as described in the Materials and Methods (section 2.16). Macrophages were incubated for 24 hours to allow adherence of the cells to the surface of a 24-well plate. After 24 hours, non-adherent cells were removed by washing and the resulting macrophage monolayer infected with either *M. smegmatis* (pSMT1) or *M. aurum* (pPA3) to obtain a 1:1 ratio of *Mycobacterium* to macrophage. The mycobacteria were incubated for four hours to allow phagocytosis; subsequently the monolayer was washed four times to remove unphagocytosed mycobacteria. Fresh medium containing rifampicin or streptomycin was then added to the monolayer and incubation continued for a further 24 hours. After 24 hours, the supernatant and monolayers were removed from the wells, lysed and the bioluminescence of lysates measured in a luminoskan luminometer after the addition of decanal. Viable counts were also done to estimate the number of extracellular and intracellular bacteria.

Mycobacterium smegmatis (pSMT1) inside macrophages and treated with 150 μg/ml rifampicin or 50 μg/ml streptomycin for 24 hours demonstrated reduced bioluminescence (Figure 3.22). For example, relative light units (RLUs) of one experiment at 24 hours were 8976 RLUs for *M. smegmatis* grown in the absence of rifampicin and 30 RLUs for *M. smegmatis* grown in the presence of rifampicin (Figure 3.22.A). There was a 0.94 log increase in the number of *M. smegmatis* grown in the absence of rifampicin and a 1.12 log decrease in the number of *M. smegmatis* grown in the presence of rifampicin (Table 3.3). Bioluminescence was also assessed in *M. smegmatis* treated with streptomycin. The relative light units of one experiment at 24 hours was 9680 RLUs for *M. smegmatis* grown in the absence of streptomycin and 131 RLUs for *M. smegmatis* grown in the presence of streptomycin (Figure 3.22.A). There was a 0.54 log increase in the number of *M. smegmatis* grown in the absence of streptomycin and a 4.25 log decrease in the number of *M. smegmatis* grown in the presence of streptomycin (Table 3.3). Thus, bioluminescence can be used to evaluate the activity of antimycobacterial agents against *M. smegmatis* in macrophages.

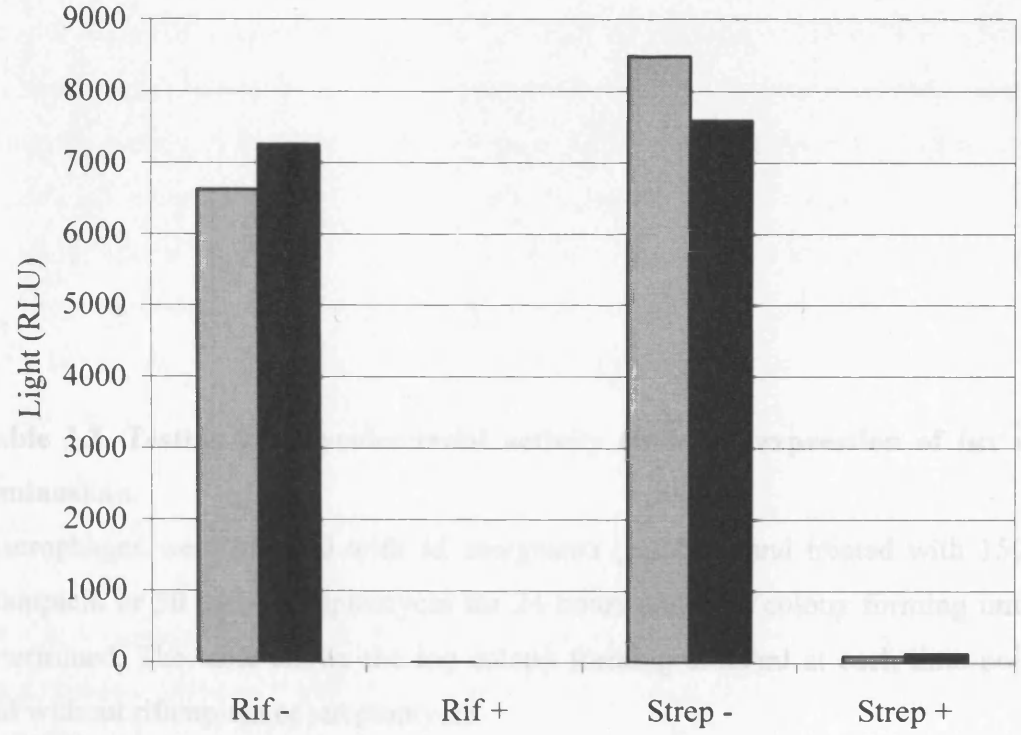
Figure 3.22. Testing antimycobacterial activity from the expression of *lux* using a luminoskan.

Macrophages were infected with *M. smegmatis* (pSMT1) and treated with 150 μg/ml rifampicin (Rif +) or 50 μg/ml streptomycin (Strep +) for 24 hours and then light was measured using the luminskan. Graph A shows the amount of light produced by *M. smegmatis* associated with the macrophage with (+) and without (-) antibiotic treatment and graph B shows the amount of light produced by *M. smegmatis* associated with the supernatant with (+) and without (-) antibiotic treatment. Graph A and B each show the results of two experiments.

A


14000
12000
10000
8000
6000
4000
2000
0
Light (RLU)
Rif -
Rif +
Strep -
Strep +

B


9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Light (RLU)
Rif -
Rif +
Strep -
Strep +

	Time (hours)	Macrophage	Supernatant
	0	7.09	7.72
Without Rifampicin	24	8.03	7.90
With Rifampicin	24	5.97	4.78

	Time (hours)	Macrophage	Supernatant
	0	7.25	7.39
Without Streptomycin	24	7.79	8.09
With Streptomycin	24	3.00	2.60

Table 3.3. Testing antimycobacterial activity from the expression of *lux* using a luminoskan.

Macrophages were infected with *M. smegmatis* (pSMT1) and treated with 150 μg/ml rifampicin or 50 μg/ml streptomycin for 24 hours and then colony forming units were determined. The table shows the log colony forming units/ml at each time point with and without rifampicin or streptomycin.

The number of treated and untreated *M. smegmatis* (pSMT1) present in the supernatant was also assessed by bioluminescence and viable counts (Figure 3.22.B). It appears that there was a large number of *M. smegmatis* present in the supernatant as seen by the high levels of light production and in the readings taken from the colony forming units (Table 3.3). The monolayers had been washed four times and as previously seen in Figure 3.21 this would be effective in removing the majority of the extracellular mycobacteria. This result is possibly due to the loss of heavily infected macrophages from the monolayer. In the absence of rifampicin or streptomycin, an increase in bioluminescence and viable counts was seen with *M. smegmatis* associated with the supernatant. In the presence of 150 μg/ml rifampicin or 50 μg/ml streptomycin a decrease in bioluminescence and viable counts was seen with *M. smegmatis* associated with the supernatant.

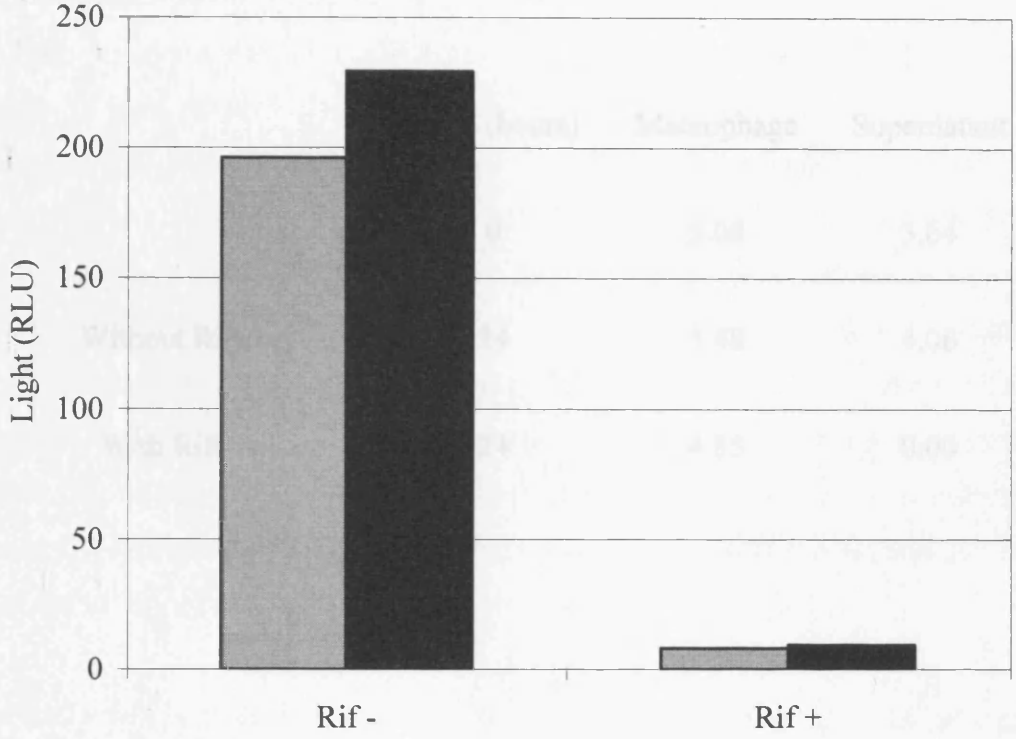
In a separate experiment macrophages infected with *M. aurum* (pPA3) and treated with 150 μg/ml rifampicin, also demonstrated reduced bioluminescence (Figure 3.23.A). For example, relative light units (RLUs) of one experiment at 24 hours was 229 RLUs for *M. aurum* in the absence of rifampicin and 9 RLUs for *M. aurum* in the presence of rifampicin. This reflects the result after colony counting. There was a 0.4 log increase in the number of *M. aurum* grown in the absence of rifampicin and a 0.23 log decrease in the number of *M. aurum* grown in the presence of rifampicin (Table 3.4). The number of treated and untreated *M. aurum* present in the supernatant was also assessed by bioluminescence and viable counts (Figure 3.23.B. and Table 3.4). In this case, the number of mycobacteria associated with the supernatant was far less than was the situation with *M. smegmatis*, as demonstrated by the very low levels of light production and from the viable counts. Of the total number of mycobacteria, less than 4% of them were associated with the supernatant. Overall, the experiment with *M. smegmatis* and *M. aurum* showed that recombinant strains of mycobacteria containing the *luxAB* genes can be used to determine the activity of antimycobacterial agents against mycobacteria in macrophages.

The macrophage assay was then used to measure antimycobacterial activity in macrophages infected with *M. aurum* (pPA3) using serial doubling dilutions of amikacin, pyrazinamide and rifampicin to achieve final concentrations ranging from

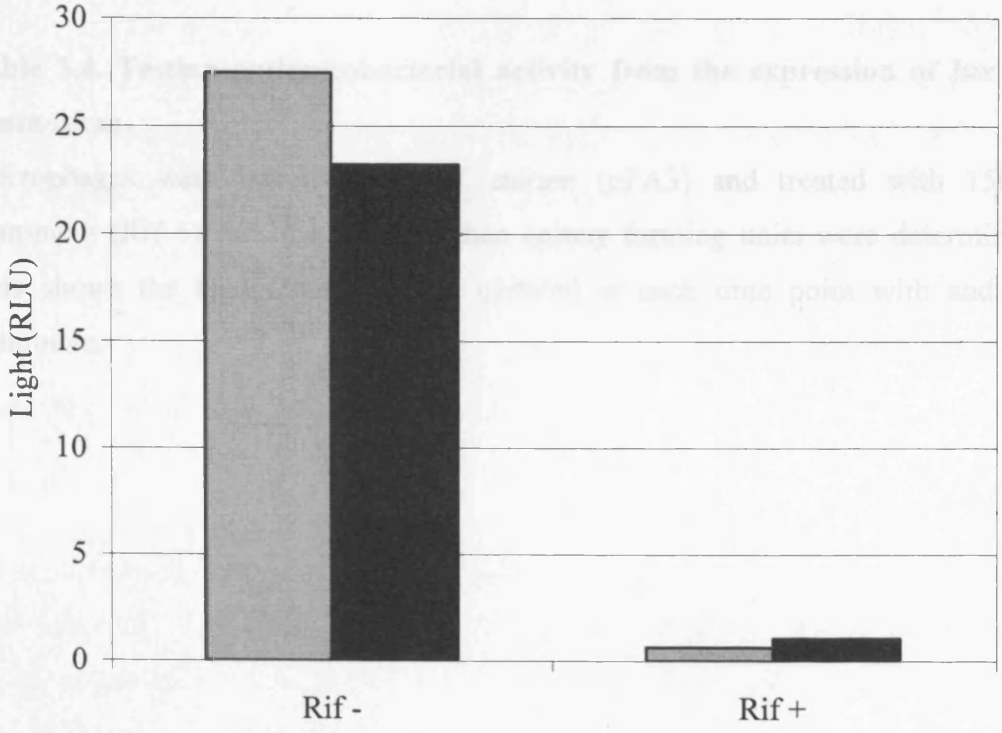
Figure 3.23. Testing antimycobacterial activity from the expression of *lux* using a luminoskan.

Macrophages were infected with *M. aurum* (pPA3) and treated with 150 μg/ml rifampicin (Rif +) for 24 hours and then light was measured with a luminoskan. Graph A shows the amount of light produced by *M. aurum* associated with the macrophage with (+) and without (-) treatment with rifampicin (Rif) and graph B shows the amount of light produced by *M. aurum* associated with the supernatant with (+) and without (-) treatment with rifampicin. Graph A and B each show the results of two experiments.

A


250
200
150
100
50
0
Light (RLU)
Rif -
Rif +

B


30
25
20
15
10
5
0
Light (RLU)
Rif -
Rif +

	Time (hours)	Macrophage	Supernatant
	0	5.08	3.64
Without Rifampicin	24	5.48	4.06
With Rifampicin	24	4.85	0.00

Table 3.4. Testing antimycobacterial activity from the expression of *lux* using a luminoskan.

Macrophages were infected with *M. aurum* (pPA3) and treated with 150 μg/ml rifampicin (Rif +) for 24 hours and then colony forming units were determined. The table shows the log colony forming units/ml at each time point with and without rifampicin.

250 μg/ml to 0.01 μg/ml. Three experiments were done on separate occasions. After infection, monolayers were incubated for 24 hours with each drug before removing the monolayers and supernatant and measuring bioluminescence. The graphs show the amount of light produced, expressed as RLUs, by *M. aurum* (pPA3) in the presence of amikacin, pyrazinamide and rifampicin at the specified concentrations (Figure 3.24.a., Figure 3.24.b., Figure 3.25.a., Figure 3.25.b., Figure 3.26.a., and Figure 3.26.b). The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graphs to show the highest and lowest points in the RLUs and which also include the average RLUs. Figure 3.24.a., Figure 3.25.a., and Figure 3.26.a., correspond to testing for antimycobacterial activity against mycobacteria within macrophages. Figure 3.24.b., Figure 3.25.b., and Figure 3.26.c., correspond to testing for antimycobacterial activity against mycobacteria in the supernatant removed from the wells that contained the treated monolayer.

Overall, when considering the three Figures, the results appear to be in agreement (Figure 3.24.a., Figure 3.25.a., and Figure 3.26.a). That is, the antimycobacterial activity against *M. aurum* (pPA3) using amikacin, pyrazinamide or rifampicin seems to be similar in the three experiments. The minimum inhibitory concentration (MIC) in this experiment is being defined as the concentration of antibiotic that gives more than fifty percent inhibition when compared to the control. The control is being taken as the lowest value of the RLUs (horizontal line) in each Figure when MIC is measured. The MIC for amikacin against *M. aurum* for the three experiments was 16 μg/ml, 63 μg/ml and 16 μg/ml (Figure 3.24.a., Figure 3.25.a., and Figure 3.26.a). The MIC for pyrazinamide against *M. aurum* for the three experiments was >16 μg/ml (highest concentration used in this one experiment), 250 μg/ml and 250 μg/ml (Figure 3.24.a., Figure 3.25.a., and Figure 3.26.a). The MIC for rifampicin against *M. aurum* for the three experiments was 2 μg/ml, 2 μg/ml and 1 μg/ml (Figure 3.24.a., Figure 3.25.a., and Figure 3.26.a).

Bioluminescence was also measured from *M. aurum* (pPA3) in the supernatant which was removed from the wells that contained monolayers treated with amikacin, pyrazinamide or rifampicin for 24 hours (Figure 3.24.b., Figure 3.25.b., and Figure 3.26.b). The findings are similar to those observed with *M. aurum* associated with the

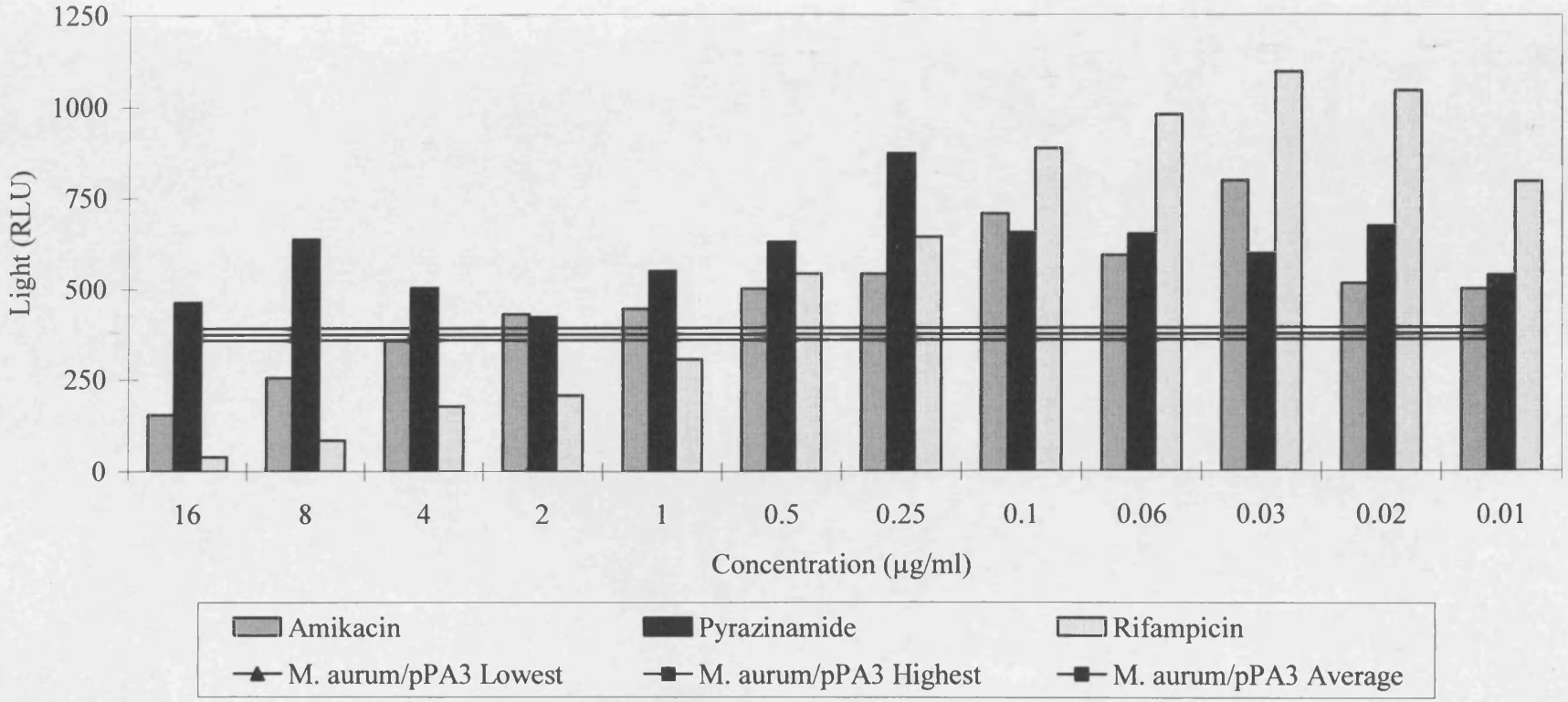


Figure 3.24.a. Testing antimycobacterial activity against intracellular mycobacteria from the expression of *lux* using a luminoskan. Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the monolayer was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the macrophage. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

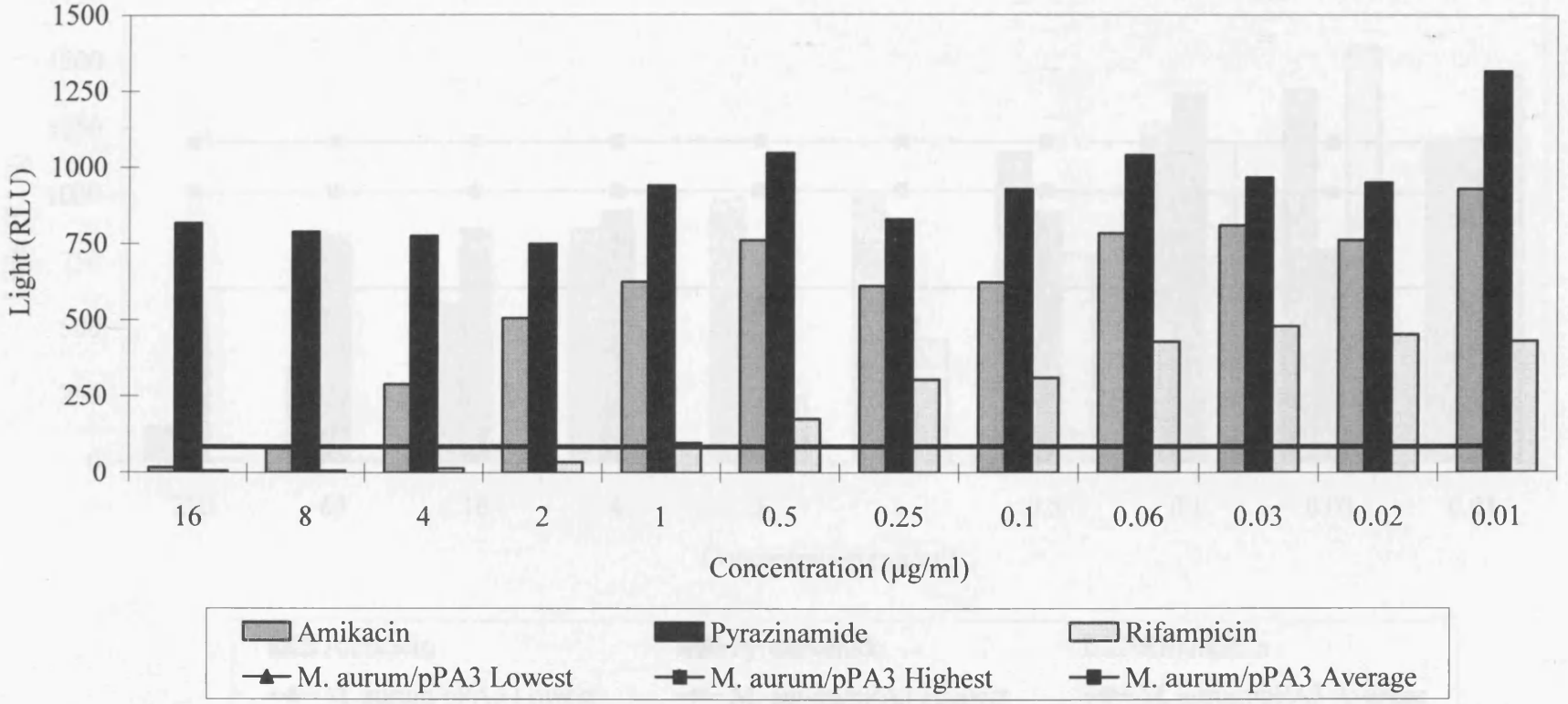


Figure 3.24.b. Testing antimycobacterial activity against extracellular mycobacteria from the expression of *lux* using a luminoskan. Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the supernatant was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the supernatant. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

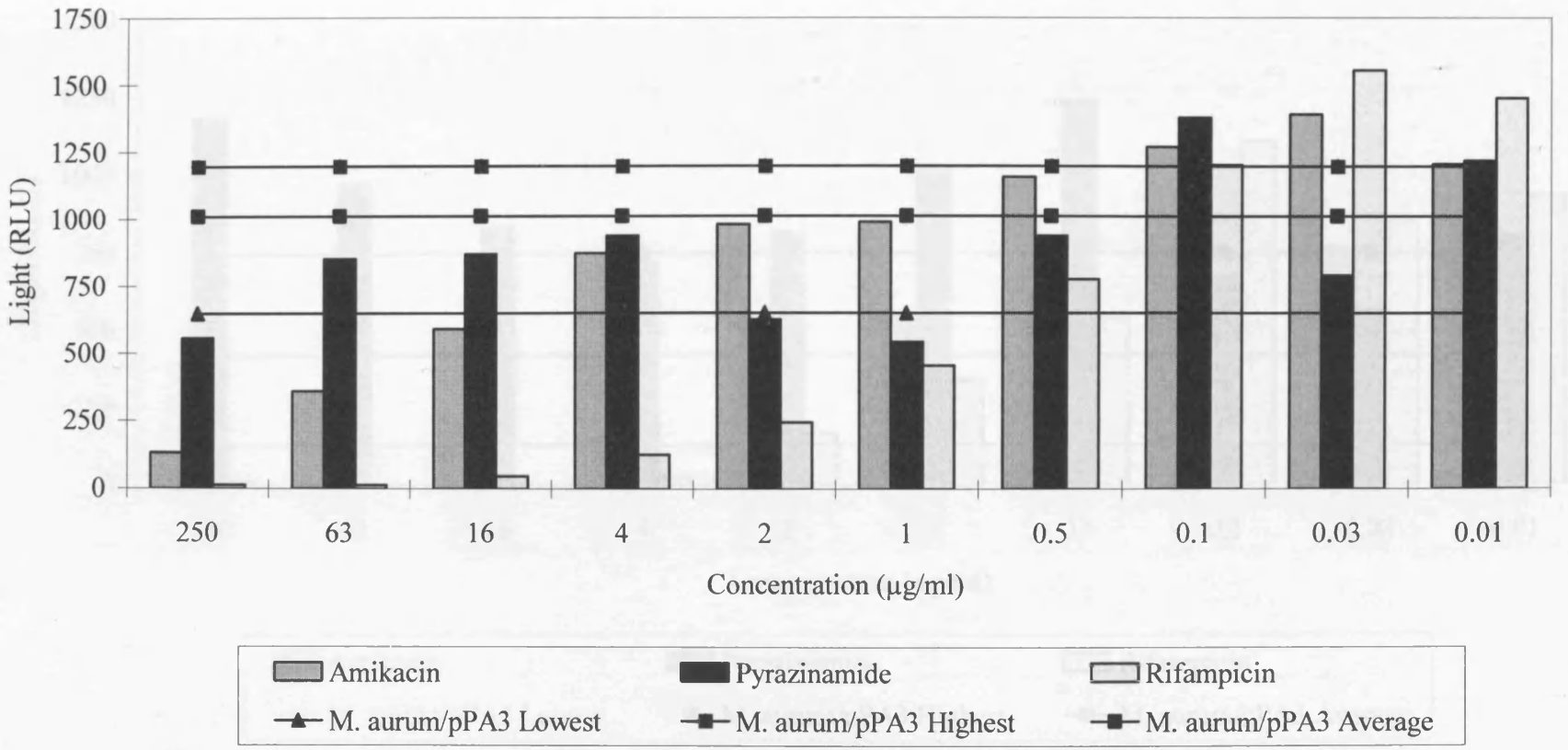


Figure 3.25.a. Testing antimycobacterial activity against intracellular mycobacteria from the expression of *lux* using a luminoskan. Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the monolayer was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the macrophage. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

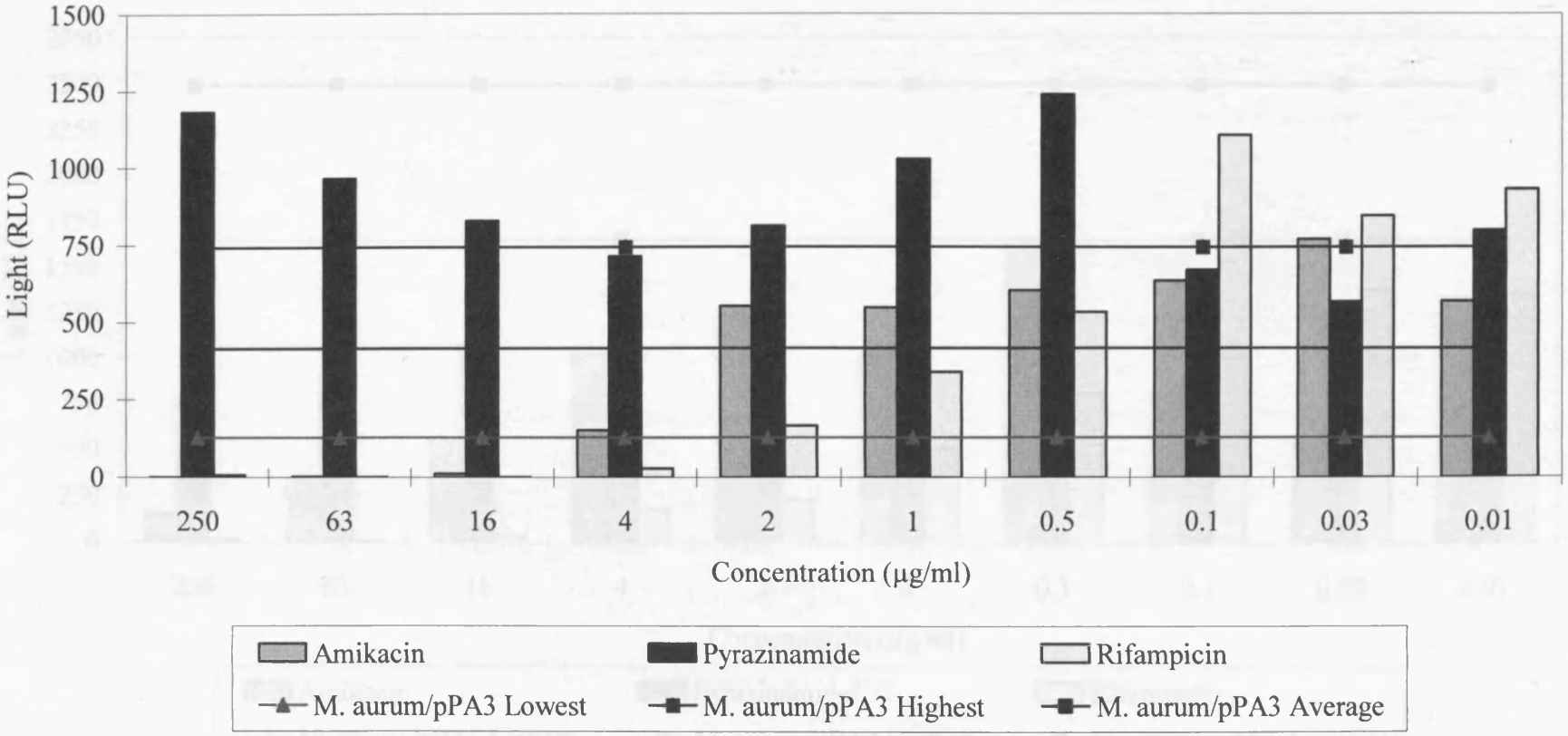


Figure 3.25.b. Testing antimycobacterial activity against extracellular mycobacteria from the expression of *lux* using a luminoskan. Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the supernatant was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the supernatant. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

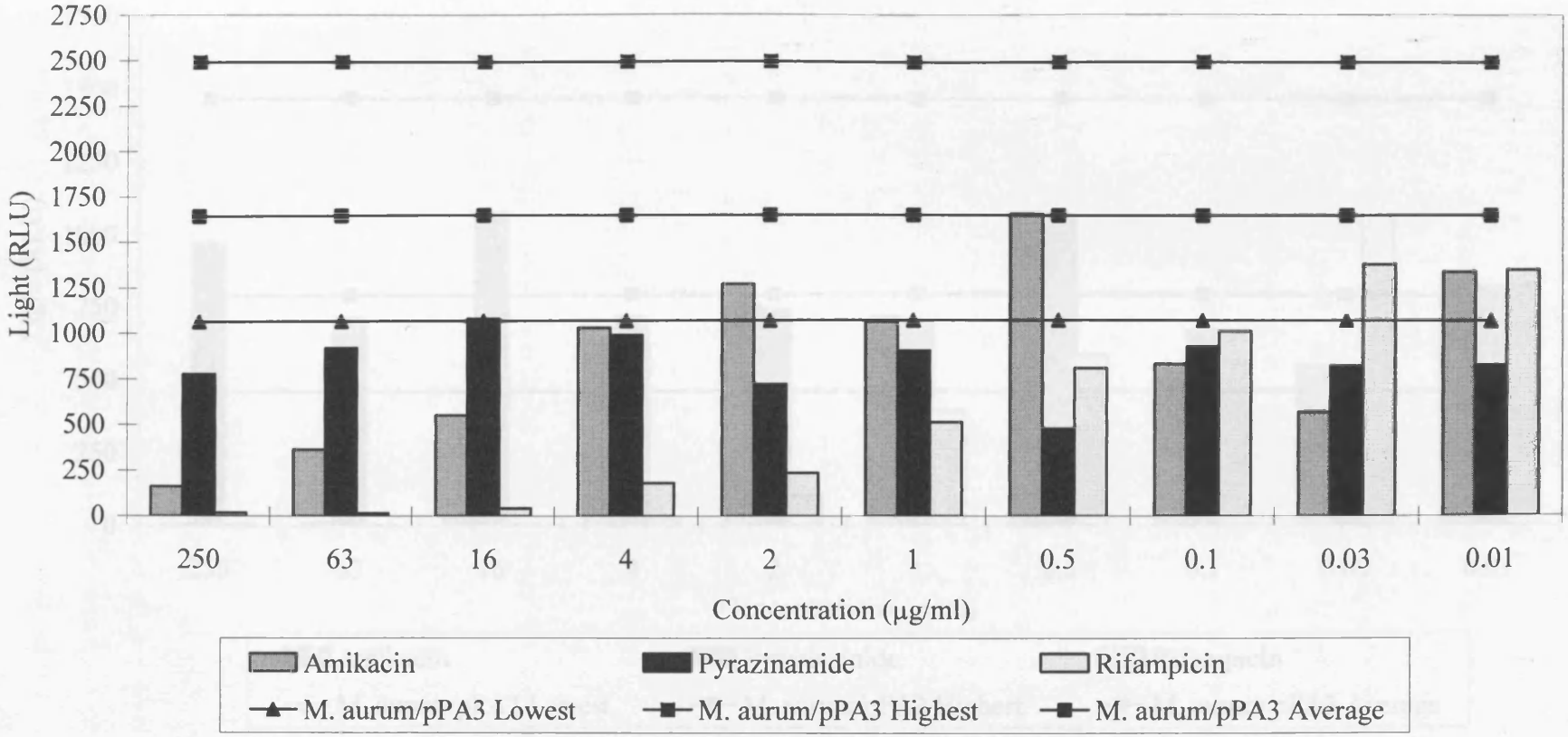


Figure 3.26.a. Testing antimycobacterial activity against intracellular mycobacteria from the expression of *lux* using a luminoskan.
Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the monolayer was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the macrophage. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

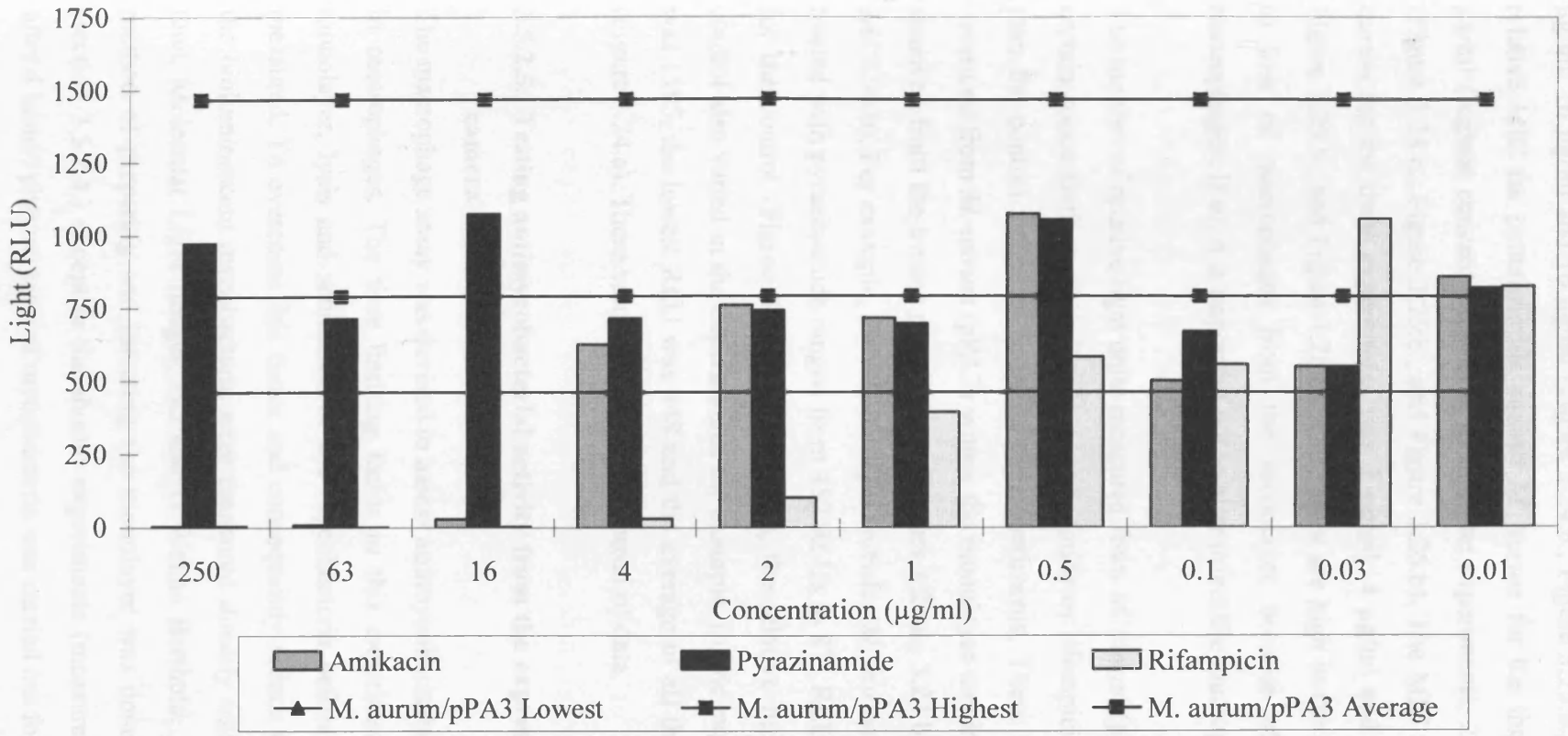


Figure 3.26.b. Testing antimycobacterial activity against extracellular mycobacteria from the expression of *lux* using a luminoskan. Macrophages were infected with *M. aurum* (pPA3) and incubated for 24 hours with amikacin, pyrazinamide or rifampicin in concentrations ranging from 250 µg/ml to 0.01 µg/ml. After incubation with each drug, the supernatant was removed and measured for bioluminescence. The graph shows the amount of light produced by *M. aurum* (pPA3) associated with the supernatant. The control, *M. aurum* (pPA3), incubated in the absence of antibiotic has been shown as three horizontal lines across the graph to show the variability of the highest (one value) and lowest (one value) points in the relative light units (RLUs) and the average RLUs (six values). All other values are the mean of two counts taken from two samples.

macrophages. The MIC for amikacin against *M. aurum* for the three experiments was 16 μg/ml, 16 μg/ml and 16 μg/ml (Figure 3.24.b., Figure 3.25.b., and Figure 3.26.b). The relative MIC for pyrazinamide against *M. aurum* for the three experiments was >16 μg/ml (highest concentration used in this one experiment), 250 μg/ml and 250 μg/ml (Figure 3.24.b., Figure 3.25.b., and Figure 3.26.b). The MIC for rifampicin against *M. aurum* for the three experiments was 2 μg/ml, 4 μg/ml and 2 μg/ml (Figure 3.24.b., Figure 3.25.b., and Figure 3.26.b). The values are high in the supernatant, possibly due to loss of macrophages from the monolayer because of over infection of the macrophages. If so, it is important not to over infect the macrophages.

The number of relative light units measured from *M. aurum* (pPA3) after treatment with certain concentrations of amikacin, pyrazinamide or rifampicin was found to be higher than the control values in some of the experiments. These included both the values measured from *M. aurum* (pPA3) within the monolayer and in the supernatant that was removed from the treated monolayers (Figures 3.24.a., 3.24.b., 3.25.a., 3.25.b., 3.26.a., and 3.26.b). For example, the relative light units for *M. aurum* (pPA3) in the monolayer treated with pyrazinamide ranged from 452 RLUs to 871 RLUs compared to 375 RLUs for the control (Figure 3.24.a). In addition, the relative light units measured in the control also varied in the experiments, for example, in one experiment the highest RLU was 1195, the lowest RLU was 648 and the average of all the values was 1009 RLUs (Figure 3.24.a). These values represent the spread of data.

3.5.2.5. Testing antimycobacterial activity from the expression of *lux* using a CCD camera.

The macrophage assay was devised to assess antimycobacterial activity of mycobacteria in macrophages. The time limiting factor in this experiment is the removal of the monolayer, lysis and sonication of the mycobacteria before bioluminescence can be measured. To overcome this factor and consequently reduce time, the wells containing the bioluminescent mycobacteria were measured directly using a CCD camera (Night Owl, Molecular Light Imager, EG and G Wallac Berthold, Wildbad, Germany). The method of preparing and infecting the monolayer was done as previously described (section 3.5.2.4.) except in the shorter experiments (measurement of bioluminescence after 4 hours) phagocytosis of mycobacteria was carried out for only 2 hours due to time restrictions. Before measurement the extracellular mycobacteria were removed and

fresh medium added. Decanal was added to the lid of a 24-well plate and the monolayer exposed to the decanal for a few minutes. Readings were measured at 490 nm using the CCD camera and measurement was set for 60 second counting with 5x5 pixel binning. Each well was measured and recordings taken. The effect of 150 μg/ml rifampicin or 100 μg/ml streptomycin was measured on macrophages infected with either *M. smegmatis* (pSMT1) or *M. aurum* (pPA3) with incubation for 4 hours (Figure 3.27., and Figure 3.28). Rifampicin and streptomycin demonstrated antimycobacterial activity against *M. smegmatis* (pSMT1) and *M. aurum* (pPA3) as seen by reduced bioluminescence (Figure 3.27., and Figure 3.28). For example, in one experiment the light units (LUs) for *M. smegmatis* of one experiment at 4 hours was 78124 LUs in the presence of rifampicin, 102220 in the presence of streptomycin and 198044 LUs in the absence of antibiotic (Figure 3.27). For *M. aurum*, the light units of one experiment at 4 hours was 1768 LUs in the presence of rifampicin, 1184 LUs in the presence of streptomycin and 6820 LUs in the absence of antibiotic (Figure 3.28). Therefore this method could detect antimycobacterial activity but with much reduced time and handling than previously required for assessment of bioluminescence.

To identify the sensitivity of the CCD camera, macrophages were infected with different numbers of bioluminescent mycobacteria. The macrophage monolayer was infected with approximately 10^4, 10^5 or 10^6 *M. aurum* (pPA3) and treated with 150 μg/ml rifampicin and incubated for either 4 hours or 24 hours, subsequently, bioluminescence was measured using the CCD camera (Figure 3.29). Results show that increasing the number of bacteria, in turn, increases the amount of light detected both at 4 hours and 24 hours. For example, 4 hours after infection, the amount of light units produced in 10^4 mycobacteria was 414 LUs, 1458 LUs were produced in 10^5 mycobacteria and 5238 LUs were produced in 10^6 mycobacteria (Figure 3.29.A). Thus, *M. aurum* at a concentration of 10^4 could be detected 4 hours after infection, however, 24 hours after infection, 1477 LUs were produced (Figure 3.29.B). The number of light units detected at 24 hours is higher than at 4 hours. The addition of rifampicin to the monolayer results in a decrease in bioluminescence at all mycobacterial concentrations and at both time points (Figure 3.29).

Experiments after this point could not be continued because the Night Owl CCD camera was only available for a limited period of time.

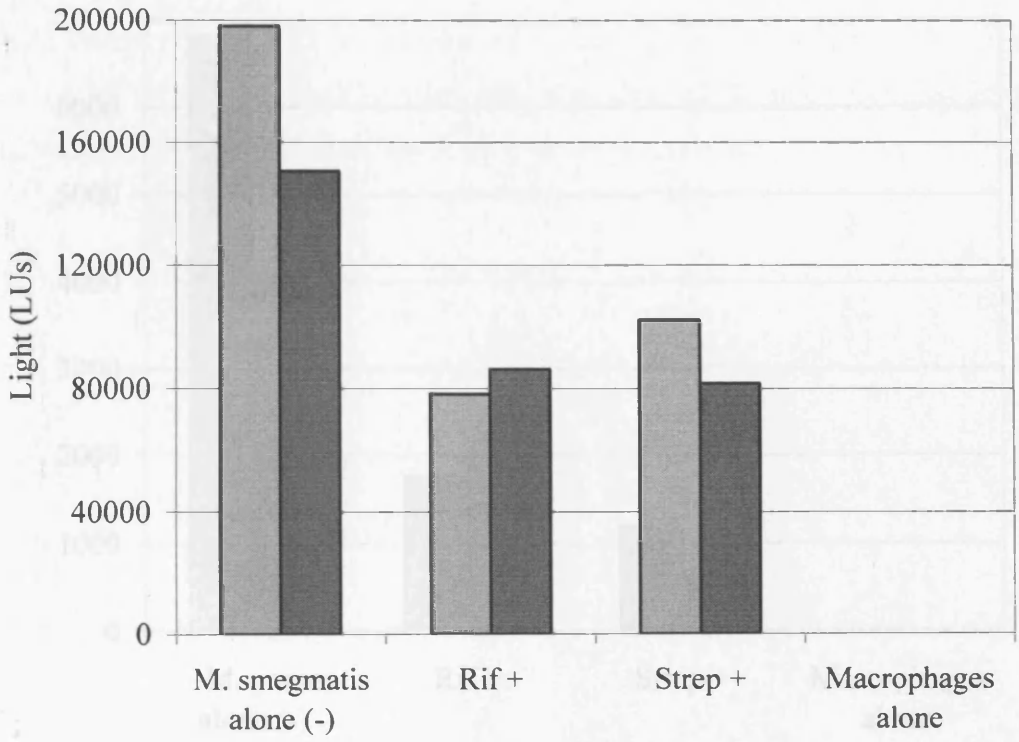


Figure 3.27. Testing antimycobacterial activity from the expression of *lux* using a CCD camera.

Macrophages were infected with *M. smegmatis* (pSMT1) and treated with 150 μg/ml rifampicin (Rif +) or 50 μg/ml streptomycin (Strep +) for 4 hours and then light was measured at 490 nm using the Night Owl Molecular Light Imager with measurement set for 60 second counting. The graph shows the amount of light produced by intracellular *M. smegmatis* with (+) and without (-) antibiotic treatment. The graph shows the results of two individual experiments.

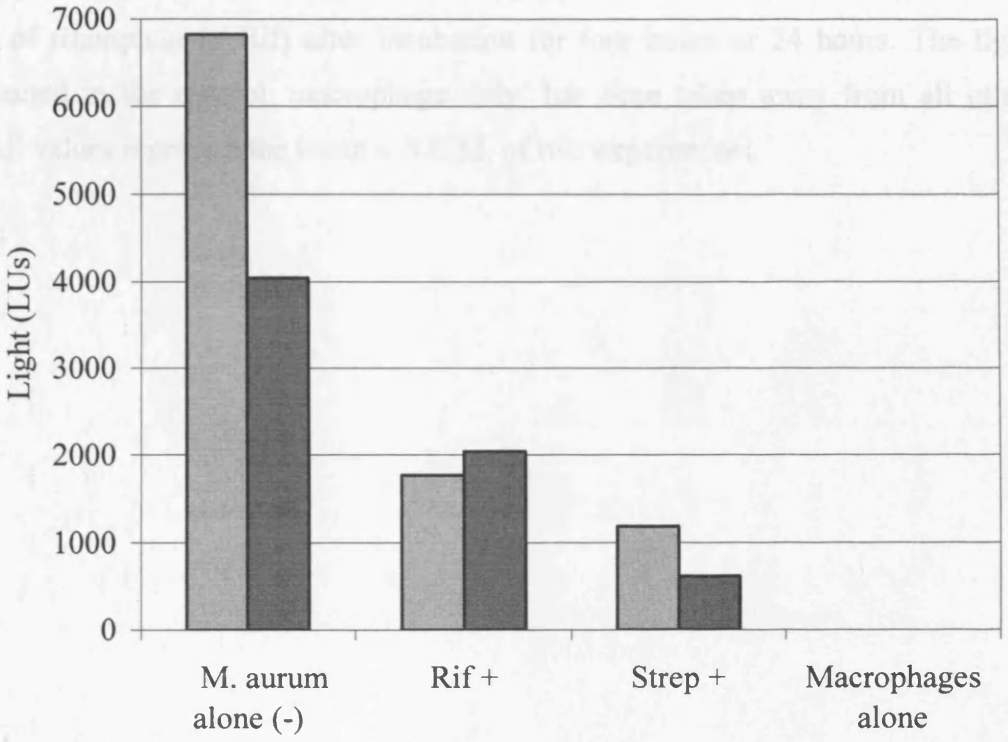


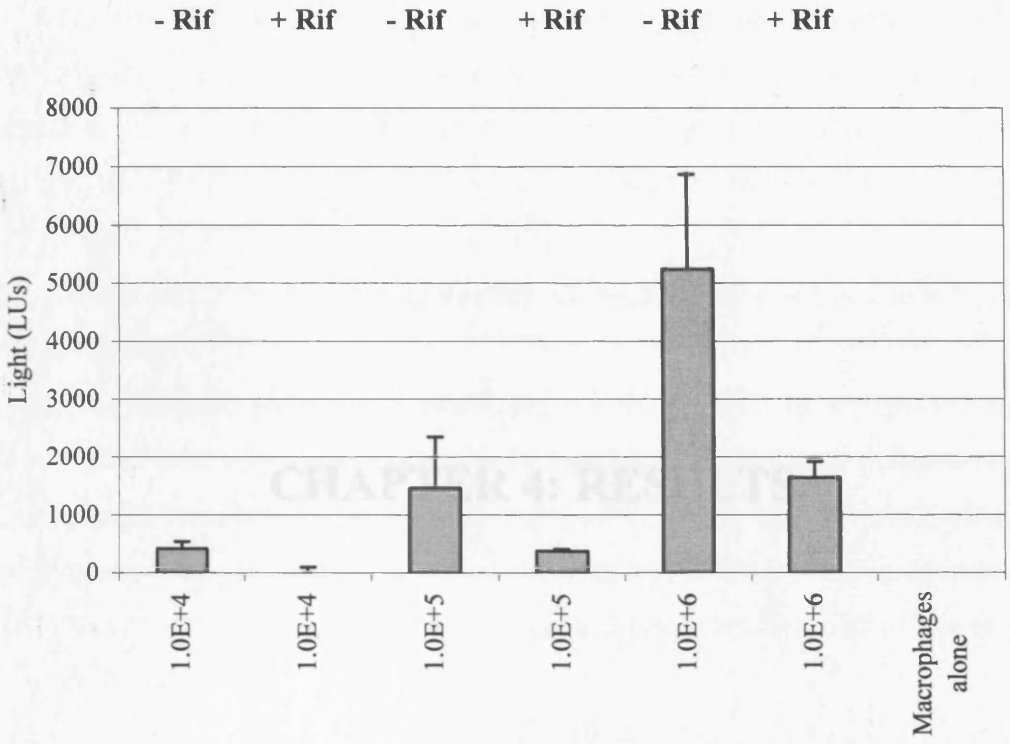
Figure 3.28. Testing antimycobacterial activity from the expression of *lux* using a CCD camera.

Macrophages were infected with *M. aurum* (pPA3) and treated with 150 μg/ml rifampicin (Rif +) or 50 μg/ml streptomycin (Strep +) for 4 hours and then light was measured at 490 nm using the Night Owl Molecular Light Imager with measurement set for 60 second counting. The graph shows the amount of light produced by intracellular *M. aurum* with (+) and without (-) antibiotic treatment. The graph shows the results of two individual experiments.

Figure 3.29. Identifying the sensitivity of the CCD camera.

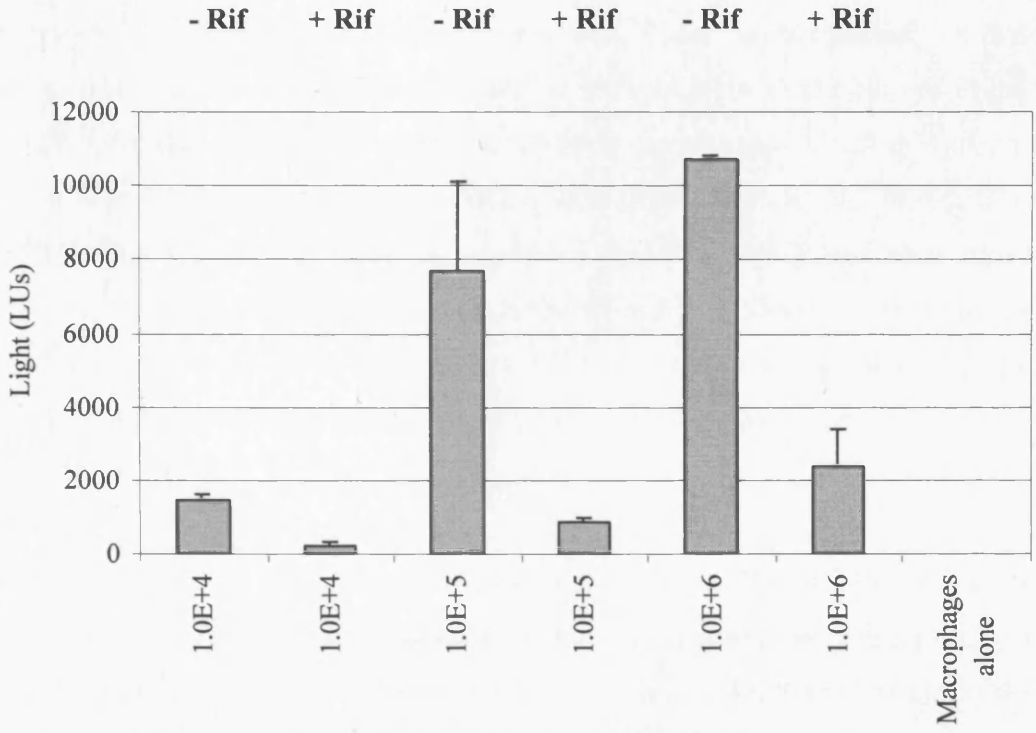
Macrophages were infected with 10^4, 10^5 or 10^6 *M. aurum* (pPA3). Graph A and graph B show the amount of light produced (Light units - LUs) by *M. aurum* (pPA3) infected macrophages. They also show the amount of light produced in the absence (- Rif) and presence of rifampicin (+ Rif) after incubation for four hours or 24 hours. The light units detected in the control, macrophage only, has been taken away from all other values. All values represent the mean ± S.E.M. of two experiments.

A



Number of *M. aurum* :pPA3 after 4 hours incubation with and without antibiotic.

B



Number of *M.aurum* :pPA3 after 24 hours incubation with and without antibiotic.

CHAPTER 4: RESULTS.

4.1. Aim.

The aim of the work described in this chapter was to construct a promoter probe vector based on the *lux* reporter system and to use this vector to assay *in vitro* regulation of expression of two genes identified from *Mycobacterium tuberculosis*, a putative decarboxylase, Rv2531c and *phoPR*, which are thought to be inducible by low pH.

4.2. Identification of genes regulated by pH and/or involved in the acid tolerance response.

To identify potential genes in *M. tuberculosis* regulated by acid and/or involved in the acid tolerance response, genes known to be regulated by acid in *S. typhimurium* and *E. coli* were used to search for genes with sequence similarity to those in *M. tuberculosis*. Several genes were considered, some were found not to be present in *M. tuberculosis*, whilst others were found to have some sequence similarity. The list of genes searched are shown in Table 4.1.

Ideas of an acid tolerance response in *Mycobacterium* were based on the evidence associated with these systems in *S. typhimurium* and *E. coli*. An increasing number of acid regulated virulence factors are being identified in these bacteria, some of which appear to function in intracellular survival. There is substantial evidence from *Salmonella* and *E. coli* that inducible amino acid decarboxylases play an important part in acid tolerance contributing to the emergency maintenance of intracellular pH (Gale, 1946; Meng and Bennett, 1992a; Watson *et al*, 1992; Park *et al*., 1996; Bearso *et al*., 1997). Also the two component regulatory system PhoPQ has been proved to be important for tolerance to acid stress. Some of the genes from *Salmonella* and *E. coli* were found not to have any homologues in the *M. tuberculosis* genome sequence. These included *crp, mviA, osmY, pagC, pagD and purB*. No genes corresponding to the *pag* genes were found.

The gene *phoPR*, coding for a two-component regulator PhoPR (PhoPR is analogous to PhoPQ) was chosen for study. There were three reasons as to why this gene was chosen. Firstly, studies have shown PhoPQ from *S. typhimurium* to be important in acid stress. Secondly, because a DNA fragment containing acid-inducible elements was previously identified from *Mycobacterium smegmatis* (Gordon, 1995). This fragment was identified as being for the gene *phoPR*, coding for a two-component regulator. This was

Gene	Function
aceEF (*odp*1)	Pyruvate dehydrogenase
ada	DNA repair
adi	Arginine decarboxylase
atrB (fabF)	Fatty acid synthesis
cadA	Lysine decarboxylase
cadB	Transport protein
cadC	Regulator
crp	Regulator
fur	Iron Regulator
gpi	Glucose-6-phosphate isomerase
gadA	Glutamate decarboxylase-alpha
gadB	Glutamate decarboxylase-beta
icd	Isocitrate dehydrogenase
mviA	Mouse virulence (Regulate RpoS)
osmY	Unknown
pagC	Virulence factor within macrophage
pagD	Virulence factor within macrophage
phoB	Regulator
phoPQ	Two-component regulator
purB	Adenylosuccinate lyase
rpoS	Sigma factor
dcor	Ornithine decarboxylase

Table 4.1. Genes regulated by pH and/or involved in the acid tolerance response. These genes have been identified from bacteria, such as *S. typhimurium* and *E. coli* and matched against the *M. tuberculosis* H37Rv genome sequence at The Sanger Centre. Genes that showed significant sequence similarity to the sequence of *M. tuberculosis* were chosen for further analysis.

done by placing fragments of *M. smegmatis* genomic DNA upstream of the promoterless luciferase genes to form a promoter probe library. The clones were exposed to acidic pH and bioluminescence was measured. Clones that expressed light under the acidic conditions would be expressing luciferase from an acid-inducible promoter. A 2.2 Kb fragment from *M. smegmatis* was found to be acid-inducible. The complete sequence was translated into protein sequence and checked against the protein database to see if any sequences were similar. One open reading frame (ORF-1) was identified as having sequence similarity with PhoR of *Bacillus subtilus* and *E. coli* (Gordon, 1995). Thirdly, although the promoter for this gene (ORF-1) was not contained in this fragment and therefore the promoter must have been located downstream of this ORF-1, it was felt that this region may be involved in acid tolerance. After showing these results to our industrial collaborators, they were in agreement for us to pursue this two-component system in this area of work.

The DNA fragment from *M. smegmatis* (ORF-1) was used to search against the *M. tuberculosis* genome sequence (http://www.sanger.ac.uk). The results of the blast search of ORF-1 from *M. smegmatis* against *M. tuberculosis* sequence are shown in Figure 4.1., and Figure 4.2. Two cosmids were found with homology to that of ORF-1 from *M. smegmatis*, MTCY10G2 and MTCY369. MTCY10G2 was found to be a probable sensor-like histidine kinase (MTCY10G2.17) that was part of an unknown two-component regulator and MTCY369 was identified as *phoR* (MTCY369.03) that was part of the two-component regulator, PhoPR.

Of the genes searched, one other group of genes was chosen for further analysis against the *M. tuberculosis* genome sequence (Table 4.2). These were the inducible decarboxylases that included, *adi* encoding arginine decarboxylase, *cadA* encoding lysine decarboxylase and *dcor* encoding ornithine decarboxylase from *E. coli*. The results of one blast search, *cadA* from *E. coli* against *M. tuberculosis* sequence is shown in Figure 4.3., and Figure 4.4. A blast search was also done using the identified protein sequence of the decarboxylase from *M. tuberculosis* (Blast program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991) and sequence similarity to *E. coli* lysine decarboxylase was found, with 38% identity and 58% similarity.

MTCY10G2

Minus Strand HSPs:

```
 Score = 604 (212.6 bits), Expect = 4.9e-59, P = 4.9e-59
 Identities = 125/266 (46%), Positives = 181/266 (68%), Frame = -3

Query:    97 PLARVSATAREVADLELERGEVRLPTPIVPVDPAVXHTEVGQLGTSXNRMLDRIASALSA 156
             PL RV+ATA EVA + L    + ++    + P D     + EVG +G + NR+LD +  AL+
Sbjct: 24040 PLRRVAATAAEVATMPLTDDDHQISVRVRPGDTDPDN-EVGIVGHTLNRLLDNVDGALAH 23864

Query:   157 RHASETRVRQFVADASHELRTPLAAIRGYTEXAQRKRGELPDDVAHAMSRVESETSRMTQ 216
             R  S+ R+RQF+ DASHELRTPLAAI+GY E  ++    +LP     +A++R+ESE  RMT
Sbjct: 23863 RVDSDLRMRQFITDASHELRTPLAAIQGYAELTRQDSSDLPPTTEYALARIESEARRMTL 23684

Query:   217 LVEDMLLLARLDAGRPLERDRVELSRLVVDTVSDRHVAGPQHKWSLDLPEDTVVIDGDEA 276
             LV+++LLL+RL  G  LE + ++L+ LV++ V+D  VA P H+W  +LP++ V ++GD A
Sbjct: 23683 LVDELLLLSRLSEGEDLETEDLDLTDLVINAVNDAAVAAPTHRWVKNLPDEPVWVNGDHA 23504

Query:   277 RLHQVMANLLXNARTHTPPGTSVTVALSADAEG----WVTVAVTDDGPGIPPELLPDVFE 332
             RLHQ+++NLL NA  HT PG +VT+ ++    G     V ++VTDDGP I PE+LP +F+
Sbjct: 23503 RLHQLVSNLLTNAWVHTQPGVTVTIGITCHRTGPNAPCVELSVTDDGPDIDPEILPHLFD 23324

Query:   333 RFARXDSSRSPREGSTGLGLAIVAAVVQ 360
             RF R   SRS   G  GLGLAIV+++V+
Sbjct: 23323 RFVRASKSRSNGSGH-GLGLAIVSSIVK 23243
```

MTCY369

Plus Strand HSPs:

```
 Score = 465 (163.7 bits), Expect = 1.9e-44, P = 1.9e-44
 Identities = 122/268 (45%), Positives = 160/268 (59%), Frame = +3

Query:    92 RRQFAPLARVSATAREVADLELERGEVRLPTPIVPVDPAVXHTEVGQLGTSXNRMLDRIA 151
             RR   PLA    TA  +   +L+R   R+P       P    TEVG+L  + N ML +I
Sbjct:  2394 RRSLRPLAEFEQTAAAIGAGQLDR---RVPQ----WHP---RTEVGRLSLALNGMLAQIQ 2543

Query:   152 SAL-----SARHA--SETRVRQFVADASHELRTPLAAIRGYTEXAQRKRGELPDDVAHAM 204
              A+     SA  A  SE R+RQF+ DASHELRTPL  IRG+ E  ++       DV   +
Sbjct:  2544 RAVASAESSAEKARDSEDRMRQFITDASHELRTPLTTIRGFAELYRQGAAR---DVGMLL 2714

Query:   205 SRVESETSRMTQLVEDMLLLARLDAGRPLERDRVELSRLVVDTVSDRHVAGPQHKWSLDL 264
             SR+ESE SRM  LV+D+LLLARLDA RPLE  RV+L  L  D   D     P+ + +L++
Sbjct:  2715 SRIESEASRMGLLVDDLLLLARLDAHRPLELCRVDLLALASDAAHDARAMDPKRRITLEV 2894

Query:   265 PED--TVVIDGDEARLHQVMANLLXNARTHTPPGTSVTVALSADAEGWVTVAVTDDGPGI 322
              +   T  + GDE+RL QV+ NL+ NA  HTP    VTV +  + +  + + V DDGPG+
Sbjct:  2895 LDGPGTPEVLGDESRLRQVLRNLVANAIQHTPESADVTVRVGTEGDDAI-LEVADDGPGM 3071

Query:   323 PPELLPDVFERFARXDSSRSPREGSTGLGLAIVAAVV 359
               E    VFERF R DSSR+   G TGLGL+IV ++V
Sbjct:  3072 SQEDALRVFERFYRADSSRARASGGTGLGLSIVDSLV 3182
```

Figure 4.1. Blast search of ORF-1 of *M. smegmatis* against the *M. tuberculosis* H37Rv database at The Sanger Centre.

Two homologues were found in *M. tuberculosis*, MTCY10G2 and MTCY369.

MTCY10G2

CDS complement (23144..24673)
/db_xref="SPTREMBL:P96368"
/note="Rv1032c, (MTCY10G2.17). Probable two-component sensor protein.

/gene="Rv1032c"
/product="hypothetical protein Rv1032c"
/translation=

"MIPDRNTRSRKAPCWRPRSLRQQLLLGVLAVVTVVLVAVGVVSVLSLSGYVT
AMNDAELVESLHALNHSYTRYRDSAQTSTPTGNLPMSQAVLEFTGQTPGNLIA
VLHDGVVIGSAVFSEDGARPAPPDVIRAIEAQVWDGGPPRVESLGSLGAYQVDS
SAAGADRLFVGVSLSLANQIIARKKVTTVALVGAALVVTAALTVWVVGYALR
PLRRVAATAAEVATMPLTDDDHQISVRVRPGDTDPDNEVGIVGHTLNRLLDNV
DGALAHRVDSDLRMRQFITDASHELRTPLAAIQGYAELTRQDSSDLPPTTEYAL
ARIESEARRMTLLVDELLLLSRLSEGEDLETEDLDLTDLVINAVNDAAVAAPTH
RWVKNLPDEPVWVNGDHARLHQLVSNLLTNAWVHTQPGVTVTIGITCHRTGP
NAPCVELSVTDDGPDIDPEILPHLFDRFVRASKSRSNGSGHGLGLAIVSSIVKAH
RGSVTAESGNGQTVFRVRLPMIEQQIATTA"

CDS complement (24681..25454)
/db_xref="SPTREMBL:Q50806"
/note="Rv1033c, (MTCY10G2.16), Probable two-component regulatory protein.

/gene="Rv1033c"
/product="hypothetical protein Rv1033c"
/translation=

"MTTMSGYTRSQRPRQAILGQLPRIHRADGSPIRVLLVDDEPALTNLVKMALHY
EGWDVEVAHDGQEAIAKFDKVGPDVLVLDIMLPDVDGLEILRRVRESDVYTPT
LFLTARDSVMDRVTGLTSGADDYMTKPFSLEELVARLRGLLRRSSHLERPADE
ALRVGDLTLDGASREVTRDGTPISLSSTEFELLRFLMRNPRRALSRTEILDRVWN
YDFAGRTSIVDLYISYLRKKIDSDREPMIHTVRGIGYMLRPPE"

MTCY369

CDS 1356..2099
/db_xref="SPTREMBL:P71814"
/note="Rv0757, (MTCY369.02)

/gene="*phoP*"
/product="PhoP"
/translation=

"MRKGVDLVTAGTPGENTTPEARVLVVDDEANIVELLSVSLKFQGFEVYTATN
GAQALDRARETRPDAVILDVMMPGMDGFGVLRRLRADGIDAPALFLTARDSL
QDKIAGLTLGGDDYVTKPFSLEEVVARLRVILRRAGKGNKEPRNVRLTFADIEL
DEETHEVWKAGQPVSLSPTEFTLLRYFVINAGTVLSKPKILDHVWRYDFGGDV
NVVESYVSYLRRKIDTGEKRLLHTLRGVGYVLREPR"

CDS 2144..3601
/db_xref="SPTREMBL:P71815"
/note="Rv0758, (MTCY369.03)

/gene="*phoR*"
/product="PhoR"
/translation=

"MARHLRGRLPLRVRLVAATLILVATGLVASGIAVTSMLQHRLTSRIDRVLLEE
AQIWAQITLPLAPDPYPGHNPDRPPSRFYVRVISPDGQSYTALNDNTAIPAVPAN
NDVGRHPTTLPSIGGSKTLWRAVSVRASDGYLTTVAIDLADVRSTVRSLVLLQV
GIGSAVLVVPGVAGYAVVRRSLRPLAEFEQTAAAIGAGQLDRRVPQWHPRTEV
GRLSLALNGMLAQIQRAVASAESSAEKARDSEDRMRQFITDASHELRTPLTTIR
GFAELYRQGAARDVGMLLSRIESEASRMGLLVDDLLLLARLDAHRPLELCRVD
LLALASDAAHDARAMDPKRRITLEVLDGPGTPEVLGDESRLRQVLRNLVANAI
QHTPESADVTVRVGTEGDDAILEVADDGPGMSQEDALRVFERFYRADSSRARA
SGGTGLGLSIVDSLVAAHGGAVTVTTALGEGCCFRVSLPRVSDVDQLSLTPVVP
GPP"

Figure 4.2. Cosmids MTCY10G2 and MTCY369.

Two cosmids were found with homology to that of ORF-1 of *M. smegmatis* and were identified from the blast search as MTCY10G2.17 and MTCY369.03. MTCY10G2.17 was found to be a probable sensor-like histidine kinase that was part of an unknown two-component regulator and MTCY369.03 was identified as *phoR* that was part of the two-component regulator, *phoPR*. The protein sequences of the two-component regulators of *M. tuberculosis* are shown.

Table 4.2. Inducible decarboxylases from *E. coli*.

Definition	Accession number	Reference
Escherichia coli arginine decarboxylase gene (*adi*)	M93362	Stim and Bennett, 1993.
Escherichia coli lysine decarboxylase gene (*cadA*)	M67452	Leveque *et al.*, 1991. Watson *et al.*, 1992.
Escherichia coli ornithine decarboxylase gene (*dcor*)	P21169	Blattner *et al.*, 1997.

Rv2531c

```
 Score = 232 (81.7 bits), Expect = 6.9e-33, Sum P(2) = 6.9e-33
 Identities = 86/298 (28%), Positives = 135/298 (45%)

Query:    58 DWDKYNLELCEEISKLNGILPLYAFANSYSTLDVSLNDLRMQVRFFEYALGAAADIARKI 117
             DW    +E  E I +L   + LY   +     +   ++  +  R F Y L    D+   +
Sbjct:   227 DW----VECAEWIRELRPHIDLYLLTDESIAAETQ-DEPDVYDRTF-YRLNDVTDLHSTV 280

Query:   118 RQNTDEYIDNILPPLTKALFKYV--PQGKYTFCTPGHMGGTAFQKSPVGSIFYDFFGPNT 175
                      +    P   AL  Y   P G++    P   G + F    +  +F+G N
Sbjct:   281 LAGLR---NRYATPFFDALRAYAAAPVGQF-HALPVARGASIFNSKSLHDMG-EFYGRNI 335

Query:   176 MKSDISISVSELGSLLDHSGPHKEAEEYIARVFNAERSYMVTNGTSTANKIVGMYSAPAG 235
               ++ S +   L SLLD  G  K A +  A  +NA ++Y VTNGTSTANKIV       G
Sbjct:   336 FMAETSTTSGGLDSLLDPHGNIKTAMDKAAVTWNANQTYFVTNGTSTANKIVVQALTRPG 395

Query:   236 STVLIDRNCHKSLTHLMMMSDITPIYFRP-TRNAYGILGGIPQSEFQHATIAKRVKETPN 294
               VLIDRNCHKS  + ++++   P+Y        Y I G +P    + A +     E
Sbjct:   396 DIVLIDRNCHKSHHYGLVLAGAYPMYLDAYPLPQYAIYGAVPLRTIKQALLDL---EAAG 452

Query:   295 ATWPVHAVI-TKSTYDGLLYGADYI-KKTLDVK-SIHF--DSAWVPYTNFSPIYQGKCGM 349
                 V  ++ T  T+DG++Y    + ++ L +K  I F  D AW  +    P  + +  M
Sbjct:   453 QLHRVRMLLLTNCTFDGVVYNPRRVMEEVLAIKPDICFLWDEAWYAFATAVPWARQRTAM 512

Query:   350 -SGDRVE 355
              + +R+E
Sbjct:   513 IAAERLE 519


 Score = 197 (69.3 bits), Expect = 6.9e-33, Sum P(2) = 6.9e-33
 Identities = 91/336 (27%), Positives = 160/336 (47%)

Query:   359 IYETQSTHKMMAAFSQASMIHVKGD----INEETFNEAYMMHTTTSPHYGIVASTETAAA 414
             +Y T STHK ++A  QASMIHV+      +  + F EA++ HT+TSP+  ++AS + A
Sbjct:   562 VYATHSTHKSLSALRQASMIHVRDQDFKALTRDAFGEAFLTHTSTSPNQQLLASLDLARR 621

Query:   415 MMKGNACKRLINGSIERAIKFRKEIKRLKSESDGWF--FDVWQ--PEHIDGAECWPLRS- 469
              +     + L+       A+ FR  +++ +  S  WF   D     P+    +     R
Sbjct:   622 QVDIEGFE-LVRHVYNMALVFRHRVRKDRLISK-WFRILDESDLVPDAFRSSTVSSYRQV 679

Query:   470 -DSAWHGFKNI-DNEHMYLDPIKVTILTPGMKKDGTMDEFGIPASLVAKYLDER-GIVVE 526
                A   +     ++   LDP ++T+       +G   +F        K L ER GI +
Sbjct:   680 RQGALADWNEAWRSDQFVLDPTRLTLFIGATGMNGY--DFR------EKILMERFGIQIN 731

Query:   527 KTGPYNLLFLFSIGIDKTKALSLPARALTEFKR-AFDLNLRVKNILPALYREAPEFYENM 585
             KT   ++L +F+IG+  +    L   L   +R A DL+   K         A
Sbjct:   732 KTSINSVLLIFTIGVTWSSVHYL----LDVLRRVAIDLDRSQKAA-----SGADLALHRR 782

Query:   586 PIQELAQNIHKLVEHHNLPDLMYRAFEVCPKMVMTPYTAFQKELHGETEEVYLE-EMVGR 644
              ++E+ Q++   L +     DL +R  +      M   +AF    + E +  Y++  + GR
Sbjct:   783 HVEEITQDLPHLPDFSEF-DLAFRPDDASSFGDMR--SAFYAG-YEEADREYVQIGLAGR 838

Query:   645 --------VNANMILPYPPGVPLVMPGEMITEESRPVLEFLQMLCEIGAHYPGFETDI 694
                     V+   ++PYPPG P+++PG+++++E   ++ FL  L     H  G+  D+
Sbjct:   839 RLAEGKTLVSTTFVVPYPPGFPVLVPGQLVSKE---IIYFLAQLDVKEIH--GYNPDL 891
```

Figure 4.3. Blast search of lysine decarboxylase (*cadA*).

Blast search of *cadA* from *E. coli* (Query) against the *M. tuberculosis* gene sequence (Sbjct) from the *Mycobacterium tuberculosis* H37Rv genome project at The Sanger Centre. A gene with sequence similarity was found in *M. tuberculosis*, Rv2531c (MTCY159).

Rv2531c (MTCY159)

CDS 16582..19425
/db_xref="SPTREMBL:P95022"
/note=" MTCY159.25. Similar to eg DCOR_ECOLI P21169 ornithine decarboxylase.

/gene="MTCY159.25"
/product="unknown"
/translation=

"MNPNSVRPRRLHVSALAAVANPSYTRLDTWNLLDDACRHLAEVDLAGLDTT
HDVARAKRLMDRIGAYERYWLYPGAQNLATFRAHLDSHSTVRLTEEVSLAVR
LLSEYGDRTALFDTSASLAEQELVAQAKQQQFYTVLLADDSPATAPDSLAECL
RQLRNPADEVQFELLVVASIEDAITAVALNGEIQAAIIRHDLPLRSRDRVPLMTT
LLGTDGDEAVANETHDWVECAEWIRELRPHIDLYLLTDESIAAETQDEPDVYD
RTFYRLNDVTDLHSTVLAGLRNRYATPFFDALRAYAAAPVGQFHALPVARGAS
IFNSKSLHDMGEFYGRNIFMAETSTTSGGLDSLLDPHGNIKTAMDKAAVTWNA
NQTYFVTNGTSTANKIVVQALTRPGDIVLIDRNCHKSHHYGLVLAGAYPMYLD
AYPLPQYAIYGAVPLRTIKQALLDLEAAGQLHRVRMLLLTNCTFDGVVYNPRR
VMEEVLAIKPDICFLWDEAWYAFATAVPWARQRTAMIAAERLEQMLSTAEYA
EEYRNWCASMDGVDRSEWVDHRLLPDPNRARVRVYATHSTHKSLSALRQAS
MIHVRDQDFKALTRDAFGEAFLTHTSTSPNQQLLASLDLARRQVDIEGFELVRH
VYNMALVFRHRVRKDRLISKWFRILDESDLVPDAFRSSTVSSYRQVRQGALAD
WNEAWRSDQFVLDPTRLTLFIGATGMNGYDFREKILMERFGIQINKTSINSVLLI
FTIGVTWSSVHYLLDVLRRVAIDLDRSQKAASGADLALHRRHVEEITQDLPHLP
DFSEFDLAFRPDDASSFGDMRSAFYAGYEEADREYVQIGLAGRRLAEGKTLVS
TTFVVPYPPGFPVLVPGQLVSKEIIYFLAQLDVKEIHGYNPDLGLSVFTQAALAR
MEAARNAVATVGAALPAFEVPRDASALNGTVNGDSVLQGVAEDA"

Figure 4.4. Cosmid Rv2531c (MTCY159).

A cosmid was found with sequence similarity to that of lysine decarboxylase (*cadA*) from *E. coli* and was identified from the blast search as MTCY159.25. MTCY159.25 was found to be similar to a decarboxylase. The protein sequence of the decarboxylase of *M. tuberculosis* is shown.

The blast search of *E. coli adi*, *cadA* and *dcor* all matched against the *M. tuberculosis* sequence Rv2531c (*Mycobacterium tuberculosis* cosmid Y159 (MTCY159.25)). The analysis of the blast searches of the other genes against *M. tuberculosis* was repeated at the end of the project and these are shown in Appendix 1. They will be discussed later in the discussion.

Overall, the two genes *phoPR* coding for a two-component regulator and Rv2531c coding for a putative decarboxylase were chosen for further study. These genes were chosen since one represented a proposed regulator, while the other was a proposed effector of acid tolerance. The hypothesis was that these two genes are involved in tolerance to acid and are acid-inducible. To identify whether the promoters for the two genes were acid-inducible and thus the genes for *phoPR* and Rv2531c are part of an acid-inducible region in *M. tuberculosis*, amplification of the 5' region immediately upstream of *phoP* and Rv2531c gene sequence and subsequent cloning upstream of a *lux* reporter would be done. This vector would then be used to assay *in vitro* regulation of expression of these two genes.

It should be noted that when this study initially took place, Rv2531c had not been annotated and therefore the gene was described as *cadA* in *M. tuberculosis* on the basis of amino acid similarity of the gene product to CadA in *E. coli*. However, the gene Rv2531c has now been designated as Rv2531c or *adi*. Results from further recent statistical analysis demonstrated that the gene product of Rv2531c was more similar to AdiA than CadA of *E. coli* (Figure 4.5). Therefore this gene will be designated as Rv2531c throughout the thesis.

```
TubercuList|>Rv2531c: adi
          Length = 947

 Score =  160 bits (401), Expect = 4e-40
 Identities = 173/683 (25%), Positives = 285/683 (41%), Gaps = 104/683 (15%)

Query: 79  LHERQQNVPVFLLGDREKALAAMDRDLLELVDEFAWILEDTADFIAGRAVAAMTRYRQQL 138
           + E + ++ ++LL D  +++AA  +D  ++ D   + L D  D  +        RY
Sbjct: 235 IRELRPHIDLYLLTD--ESIAAETQDEPDVYDRTFYRLNDVTDLHSTVLAGLRNRYAT-- 290

Query: 139 LPPLFSALMKYSDIHEYSWAAPGHQGGVGFTKTPAGRFYHDYYGENLFRTDMGIERTSLG 198
             P F AL  Y+      + A     G     + +     ++YG N+F  +       L
Sbjct: 291 --PFFDALRAYAAAPVGQFHALPVARGASIFNSKSLHDMGEFYGRNIFMAETSTTSGGLD 348

Query: 199 SLLDHTGAFGESEKYAARVFGADRSWSVVVGTSGSNRTIMQACMTDNDVVVVDRNCHKSI 258
           SLLD  G    +   AA  + A++++ V  GTS +N+ ++QA     D+V++DRNCHKS
Sbjct: 349 SLLDPHGNIKTAMDKAAVTWNANQTYFVTNGTSTANKIVVQALTRPGDIVLIDRNCHKSH 408

Query: 259 EQGLMLTGAKPVYM----VPSRNRYGIIGPIYPQEMQPETLQKKISESPLTKDKAGQ--K 312
             GL+L GA P+Y+    +P    YG + P+           + I ++ L  + AGQ  +
Sbjct: 409 HYGLVLAGAYPMYLDAYPLPQYAIYGAV-PL-----------RTIKQALLDLEAAGQLHR 456

Query: 313 PSYCVVTNCTYDGVCYNAKEAQD--LLEKTSDRLHFDEAWYGYARFNP------------ 358
             ++TNCT+DGV YN +   +  L  K      +DEAWY +A   P
Sbjct: 457 VRMLLLTNCTFDGVVYNPRRVMEEVLAIKPDICFLWDEAWYAFATAVPWARQRTAMIAAE 516

Query: 359 ---------IYADHY---------AMRGEPGDHN--------GPTVFATHSTHKLLNALS 392
                  YA+ Y           R E  DH             V+ATHSTHK L+AL
Sbjct: 517 RLEQMLSTAEYAEEYRNWCASMDGVDRSEWVDHRLLPDPNRARVRVYATHSTHKSLSALR 576

Query: 393 QASYIHVR-EGRGAINFSRFNQAYMMHATTSPLYAICASNDVAVSMMDGNSGLSLTQEVI 451
           QAS IHVR +   A+    F +A++ H +TSP   + AS D+A   +D   G  L + V
Sbjct: 577 QASMIHVRDQDFKALTRDAFGEAFLTHTSTSPNQQLLASLDLARRQVD-IEGFELVRHVY 635

Query: 452 DEAVDFRQAMARLYKEFTADGSWFFKPWNKEVVTDPQTGKTYDFADAPTKLLTTVQDCWV 511
           + A+ FR  +    ++       WF      ++V D         F  +        V+   +
Sbjct: 636 NMALVFRHRV----RKDRLISKWFRILDESDLVPDA-------FRSSTVSSYRQVRQGAL 684

Query: 512 MHPGESWHGFKDIPDNWSMLDPIKVSILAPGMGEDG-ELEETGVPAALVTAWLGRHGIVP 570
             E+W        +  +LDP ++++     G +G +  E           + R GI
Sbjct: 685 ADWNEAWR------SDQFVLDPTRLTLFIGATGMNGYDFRE--------KILMERFGIQI 730

Query: 571 TRTTDFQIMFLFSMGVTRGKWGTLVNTLCSFKRHYDANTPLAQ-----VMPELVEQYPDT 625
            +T+   ++ +F++GVT      L++ L       D +   A      +    VE+
Sbjct: 731 NKTSINSVLLIFTIGVTWSSVHYLLDVLRRVAIDLDRSQKAASGADLALHRRHVEEITQD 790

Query: 626 YANMGIHDLGDTMFAWLKENNPGARLNEAYSGLPVAEVTPREAYNAIVDNNVELVSIENL 685
             ++      D  F     ++ G   +  Y+G   A+      Y  I      L   + L
Sbjct: 791 LPHLPDFSEFDLAFRPDDASSFGDMRSAFYAGYEEAD----REYVQIGLAGRRLAEGKTL 846

Query: 686 PGRIAANSVIPYPPGIPMLLSGE 708
             ++   V+PYPPG P+L+ G+
Sbjct: 847 ---VSTTFVVPYPPGFPVLVPGQ 866
```

Figure 4.5. Blast search of lysine decarboxylase (*cadA*)

A recent blast search of *cadA* from *E. coli* (Query) against the *M. tuberculosis* gene sequence (Sbjct) from the *Mycobacterium tuberculosis* H37Rv genome project at The Sanger Centre. A gene with sequence similarity to *M. tuberculosis* Rv2531c (*adiA*) was found.

4.3. Vector Construction.

4.3.1. Construction of the promoter probe vector, pJS11.

The promoter probe vector pJS11 based on *lux* was constructed for use in mycobacteria (Figure 4.6). The *Escherichia coli-Mycobacterium* shuttle vector pOLYG was used for the basis of construction for the vector, which consisted of an origin of replication for *E. coli* and mycobacteria and a hygromycin resistance gene. The *luxAB* genes of *Vibrio harveyi* were obtained from pSB226 (Hill *et al.*, 1991). To construct the promoter probe vector, pJS11, the *luxAB* genes from pSB226, were digested with *Eco*R I and then the DNA treated with DNA polymerase I large fragment (Klenow) to end-fill the overhanging termini. Subsequently the DNA was digested with *Sma* I thus resulting in the excision of the *luxAB* fragment of approximately 2.1 Kb. The restriction fragments were separated through a 0.7% (w/v) agarose gel. The 2.1 Kb *Eco*R I/*Sma* I fragment of *luxAB* was ligated into the *Eco*R V site of pOLYG. The ligation product was introduced into *E. coli* strain DH5α by electroporation and transformants were selected on 200 μg/ml hygromycin B.

Transformants containing the *luxAB* genes were identified by adding decanal onto the lid of the Petri-dish and exposing the transformants to Cronex film for several minutes depending on the intensity of the light signal. Light and dark colonies were identified and both colony types were chosen for further investigation. DNA was prepared from each type of colony and to confirm the orientation and presence of the *luxAB* fragment in pOLYG the DNA was digested with *Pst* I. Two constructs were identified, pJS10 and pJS11. Digestion of pJS10 plasmid DNA with *Pst* I resulted in two fragments of approximate size of 7000 bp and 300 bp. Digestion of pJS11 plasmid DNA with *Pst* I resulted in three fragments of approximate size of 5500 bp, 1500 bp and 300 bp. Transformants of pJS10 resulted in colonies that were light and transformants of pJS11 resulted in dark colonies (Figure 4.7). Transformants were dark or light depending on the orientation of the *luxAB* genes in pOLYG. Transformants of pJS10 were identified as transcribing *luxAB* in the opposite direction to the hygromycin resistance gene, whilst pJS11 transformants transcribed *luxAB* in the same direction as the hygromycin resistance gene. This demonstrated successful cloning of the *luxAB* DNA fragment into pOLYG and further work was done with the promoter probe vector, pJS11.

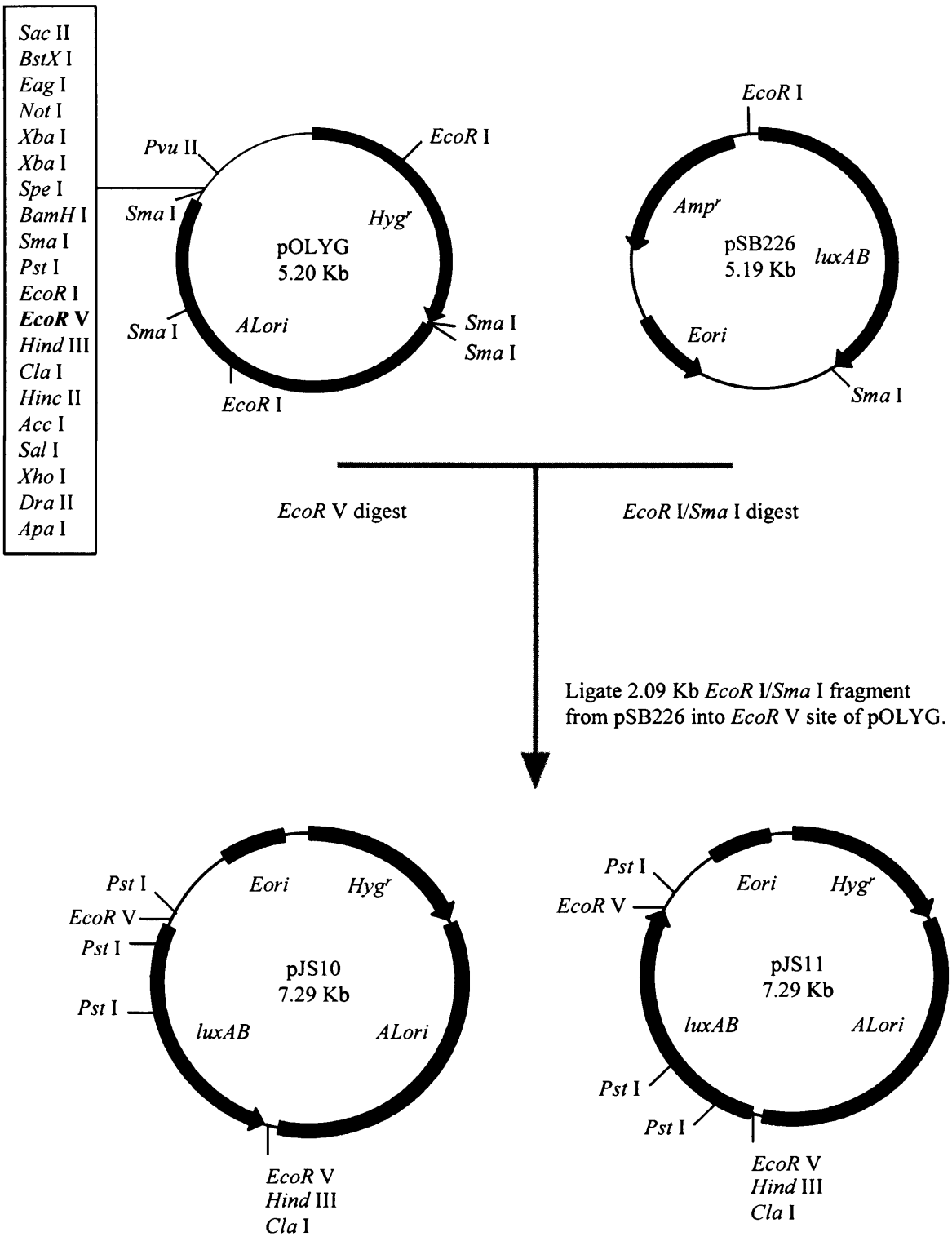


Figure 4.6. Construction of the promoter probe vector, pJS11.

The *luxAB* genes from pSB226 were digested with *Eco*R I/*Sma* I and ligated into the *EcoR* V of pOLYG to produce pJS11. The construct pJS10 was also identified but as transcribing *luxAB* in the opposite direction to the hygromycin resistance gene. Abbreviations are mycobacterial origin of replication (*ALori*), *Escherichia coli* origin of replication (*Eori*), hygromycin resistance (*Hyg*^{r}), ampicillin resistance (*Amp*^{r}) and luciferase genes (*luxAB*).

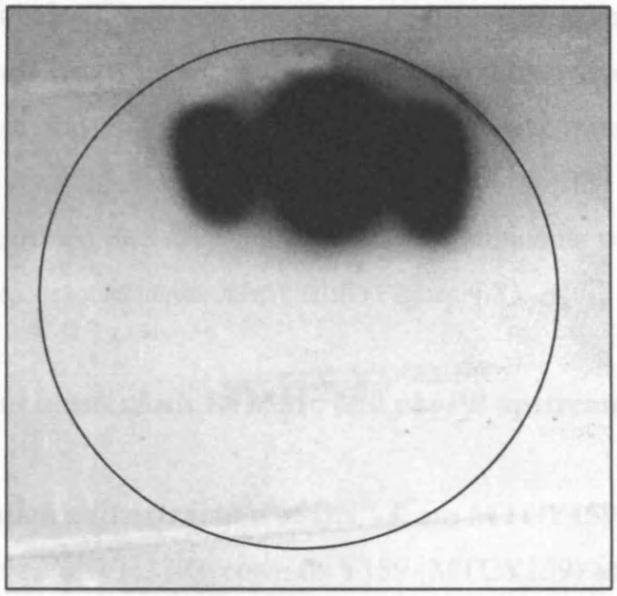


Figure 4.7. Construction of the promoter probe vector, pJS10.
Transformants containing the plasmid pJS11 were identified by exposing Cronex film to the transformants in complete darkness with the addition of aldehyde. *E. coli* containing the promoter probe vector pJS11 were dark, that is, the Cronex film was not exposed. Light was detected from *E. coli* transformed with pJS10. The X-ray film shows the light produced from *E. coli* (pJS10) with the addition of aldehyde (black streaks). (Image of a Petri-dish).

4.3.2. Transformation of pJS11 and pJS10 DNA into *Mycobacterium smegmatis* mc^2155.

Prior to continuation of further work with pJS11, to confirm that the recombinant *Mycobacterium* was indeed dark and thus there was no expression from *luxAB*, pJS11 DNA was transformed into *M. smegmatis*. The transformants were exposed to decanal in complete darkness and Cronex film overlaid for several minutes. Only very low levels of light were detected from colonies transformed with pJS11. In addition, pJS10 DNA was also transformed into *M. smegmatis* and recombinants were found to produce high levels of light, as detected with X-ray film (Figure 4.8).

4.4. *Mycobacterium tuberculosis* Rv2531c and *phoPR* upstream region.

4.4.1. Transformation and extraction of DNA from MTCY159 and MTCY369.

Mycobacterium tuberculosis H37Rv cosmids Y159 (MTCY159) and Y369 (MTCY369) carrying genes homologous to an amino acid decarboxylase, Rv2531c, and a gene homologous to a two-component regulator, *phoPR* respectively were obtained as DNA from the Institut Pasteur. To maintain the cosmids, they were transformed into *E. coli* strain DH5α by electroporation and transformants were selected on 50 μg/ml ampicillin.

4.4.2. Design of the PCR primers upstream of Rv2531c and *phoPR* from the *Mycobacterium tuberculosis* gene sequence.

To amplify the sequence containing the possible promoter region of Rv2531c (P*pd* (*pd* – putative decarboxylase)) and *phoPR* (P*phoPR*) in the *M. tuberculosis* cosmids MTCY159 and MTCY369 respectively, PCR primers were designed from the 5' region upstream of the gene sequence of Rv2531c and *phoPR* (www.sanger.ac.uk) (Figure 4.9 and Figure 4.10). Restriction endonuclease sites were incorporated into the primers to enable the amplified PCR products to be directly ligated into the multiple cloning site of pJS11. The forward primer MTCY159-F and the reverse primer MTCY159-R were designed to amplify the 301 bp region upstream of the Rv2531c gene from the cosmid MTCY159 of *M. tuberculosis* (Materials and Methods, section 2.5, Table 2.3). A *Cla* I restriction site was incorporated into the forward primer and a *Hind* III restriction site into the reverse primer. The forward primer MTCY369-F and the reverse primer MTCY369-R were designed to amplify the 1065 bp region upstream of the *phoPR* gene

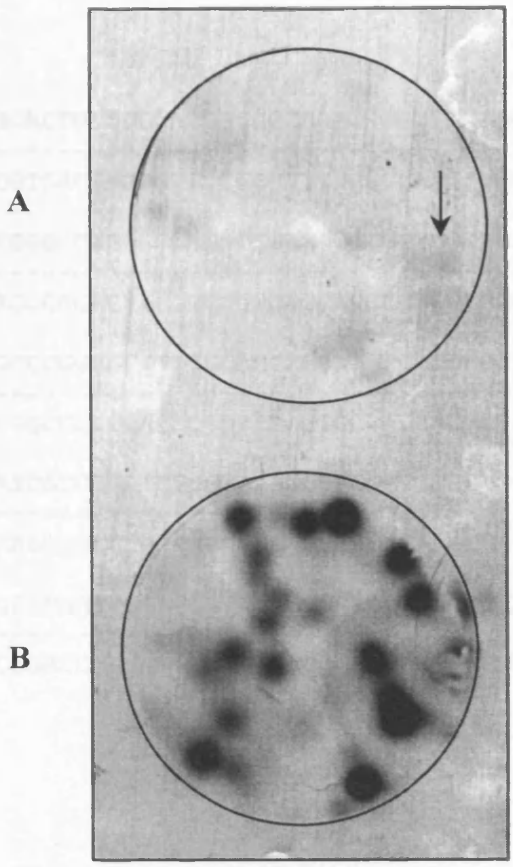


Figure 4.8. Transformation of pJS11 and pJS10 DNA into *Mycobacterium smegmatis* mc^{2}155.

pJS11 and pJS10 DNA was transformed into *M. smegmatis* and the transformants exposed to decanal in complete darkness and Cronex film overlaid for several minutes. Only very low levels of light were detected from colonies transformed with pJS11 (A – grey shading). High levels of light was detected from *M. smegmatis* transformed with pJS10 DNA (B - black dots) as detected with X-ray film. (Image of two Petri-dishes).

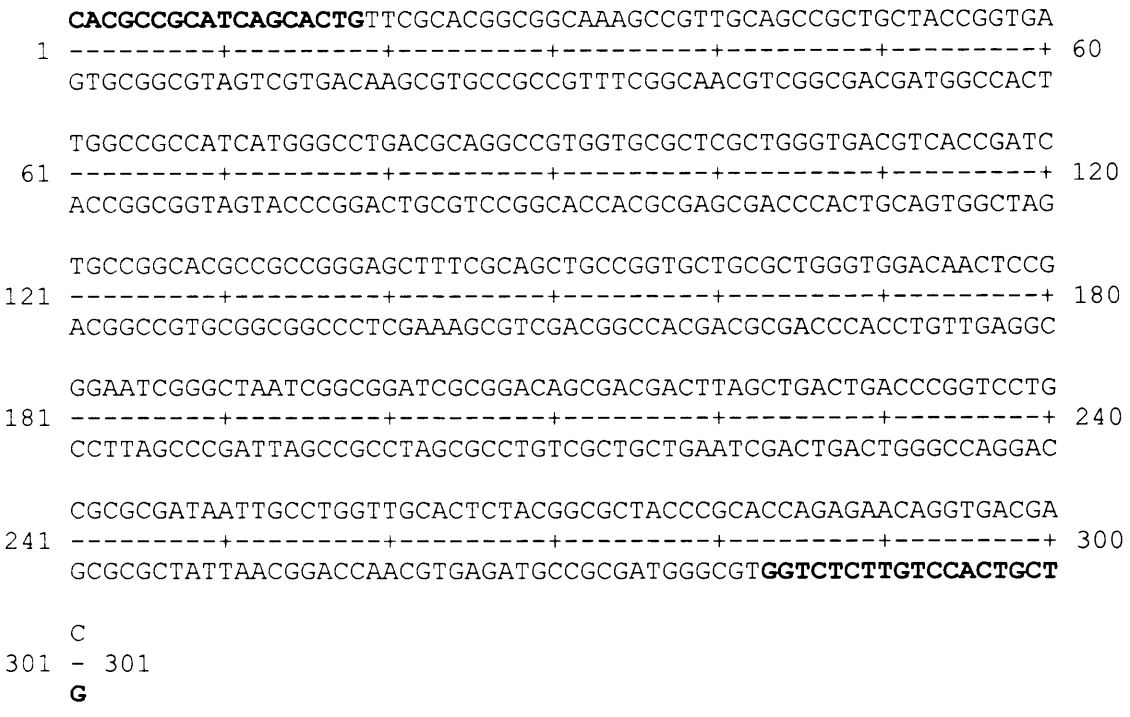


Figure 4.9. Design of the PCR primers upstream of Rv2531c from the *Mycobacterium tuberculosis* gene sequence.

The forward and reverse primers for the amplification of P*pd* are shown on the sequence in bold. This sequence is immediately upstream of the gene Rv2531c (putative decarboxylase) from *M. tuberculosis* cosmid Y159 (MTCY159). The sequence is shown 5` to 3`.


GATCAGCCGAACCAACTGTCAAGCATCAGCCGAAACATCGTCAGGCATCACCCGAACCCA
1 ---------+---------+---------+---------+---------+---------+ 60
CTAGTCGGCTTGGTTGACAGTTCGTAGTCGGCTTTGTAGCAGTCCGTAGTGGGCTTGGGT
AAACGTCAAGCATCAGCCGAGGTACTACACGAACGCTTGAGCCCCCTGTCAGGATTGAAC
61 ---------+---------+---------+---------+---------+---------+ 120
TTTGCAGTTCGTAGTCGGCTCCATGATGTGCTTGCGAACTCGGGGGACAGTCCTAACTTG
TGACGACCGCTCGCTTACAAGGCGAGTGCTCTACCACTGAGCTAAGGAGGCCGATGAAAT
121 ---------+---------+---------+---------+---------+---------+ 180
ACTGCTGGCGAGCGAATGTTCCGCTCACGAGATGGTGACTCGATTCCTCCGGCTACTTTA
CGCTGTGAGTCTAGCCGCTCACTCGCTGTCGACGACGCGTTGCGAACGCACCGACCGCGA
181 ---------+---------+---------+---------+---------+---------+ 240
GCGACACTCAGATCGGCGAGTGAGCGACAGCTGCTGCGCAACGCTTGCGTGGCTGGCGCT
CGACGAGCGGCGCGCGGGACGGCGCCCGGGCAGTGGAATGCGCTCGGCGATGCTGCTCAG
241 ---------+---------+---------+---------+---------+---------+ 300
GCTGCTCGCCGCGCGCCCTGCCGCGGGCCCGTCACCTTACGCGAGCCGCTACGACGAGTC
CGGGTTGACCACCATGGTAAGTGCGATCACAGCGTCTTGCAGCGTCGCGATGGCCGGCTC
301 ---------+---------+---------+---------+---------+---------+ 360
GCCCAACTGGTGGTACCATTCACGCTAGTGTCGCAGAACGTCGCAGCGCTACCGGCCGAG
GAGCGCCTCCATCCCGGGTGTCAGCCGCGCCAACGTGTCGGCGACGTCGGCGAGCTGTTC
361 ---------+---------+---------+---------+---------+---------+ 420
CTCGCGGAGGTAGGGCCCACAGTCGGCGCGGTTGCACAGCCGCTGCAGCCGCTCGACAAG
GAGCGGTCCGTCCTTGGCCGTTATCTTGTCGATCAGTCCGCCTTCGGCCAGCAGCCGGTC
421 ---------+---------+---------+---------+---------+---------+ 480
CTCGCCAGGCAGGAACCGGCAATAGAACAGCTAGTCAGGCGGAAGCCGGTCGTCGGCCAG
GGCCAGCCCGTCTTCGGAGAGCACCCGCTCGATAAGTCCGTCCTCGGCGAGCAGCTGGTC
481 ---------+---------+---------+---------+---------+---------+ 540
CCGGTCGGGCAGAAGCCTCTCGTGGGCGAGCTATTCAGGCAGGAGCCGCTCGTCGACCAG
GGCCAGTCCGCCCGGTTGCAGCGCGCGCTGCATGGCGCCGCCTTCAGCGGTCAGGCGGTC
541 ---------+---------+---------+---------+---------+---------+ 600
CCGGTCAGGCGGGCCAACGTCGCGCGCGACGTACCGCGGCGGAAGTCGCCAGTCCGCCAG
GAGTAAGCCGCCGGGCTGGGTCAGCAGGTCGACCACCCCGCCGGGCCGCAGCATCCGGTC
601 ---------+---------+---------+---------+---------+---------+ 660
CTCATTCGGCGGCCCGACCCAGTCGTCCAGCTGGTGGGGCGGCCCGGCGTCGTAGGCCAG
CATCGGCCCGTTGGGCGCGATGGCGCGTCCCAGCGGCATATCGTCGTCCAATAGCCTGGC
661 ---------+---------+---------+---------+---------+---------+ 720
GTAGCCGGGCAACCCGCGCTACCGCGCAGGGTCGCCGTATAGCAGCAGGTTATCGGACCG
CAGCCGGTTGGCGCGGGCAATCGTGTCATCGATTCCCAGCATGTTGGCCATTGAGGTCGA
721 ---------+---------+---------+---------+---------+---------+ 780
GTCGGCCAACCGCGCCCGTTAGCACAGTAGCTAAGGGTCGTACAACCGGTAACTCCAGCT
CCCGCTTGCGCCGCCGGCATCACCCAACGCTTGTTTGGCCATGTCAACCGCCGCGCCGGC
781 ---------+---------+---------+---------+---------+---------+ 840
GGGCGAACGCGGCGGCCGTAGTGGGTTGCGAACAAACCGGTACAGTTGGCGGCGCGGCCG
CATGTTCAAACCGGTGTCGGCGGCGGCGAGCCCCGCTCGTGCGGGCCAGGTCGCAATACC
841 ---------+---------+---------+---------+---------+---------+ 900
GTACAAGTTTGGCCACAGCCGCCGCCGCTCGGGGCGAGCACGCCCGGTCCAGCGTTATGG

```
    CACGAGGGTTTGGCCGAGGTTCATTCTGCGAGTGTATTCACGGCGCGCCGTGGATTGAGC
901 ---------+---------+---------+---------+---------+---------+ 960
    GTGCTCCCAAACCGGCTCCAAGTAAGACGCTCACATAAGTGCCGCGCGGCACCTAACTCG

    GGCAACGGTCCAAGCTGATTTGGCGATTCCTGGCAGACTGTTAGCAGACTACTGGCAACG
961 ---------+---------+---------+---------+---------+---------+ 1020
    CCGTTGCCAGGTTCGACTAAACCGCTAAGGACCGTCTGACAATCGTCTGATGACCGTTGC

     AGCTTTCAGGAATTACACAATGACTGTGAAGGTAACGTTCAACCA
1021 ---------+---------+---------+---------+----- 1065
     TCGAAAGTCCTTAATGTGTTACTGACACTTCCATTGCAAGTTGGT
```

Figure 4.10. Design of the PCR primers upstream of *phoPR* from the *Mycobacterium tuberculosis* gene sequence.

The forward and reverse primers for the amplification of P*phoPR* are shown on the 1065 bp sequence in bold. This sequence is immediately upstream of the gene *phoPR* (two-component system) from *M. tuberculosis* cosmid Y369 (MTCY369). The sequence is shown 5` to 3`.

from the cosmid MTCY369 of *M. tuberculosis* (Materials and Methods, section 2.5, Table 2.3). A *Cla* I and *Hind* III restriction site were incorporated into the forward primer and a *Cla* I restriction site was incorporated into the reverse primer. Restriction endonuclease sites were identified from the sequence using the 'MAP' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

4.4.3. PCR to amplify the promoter for the Rv2531c and *phoPR* genes.

Amplification of the 5' region upstream of the genes Rv2531c and *phoPR* was done using primers MTCY159-F/MTCY159-R and MTCY369-F/MTCY369-R (section 2.5, Table 2.3.) respectively using the polymerase chain reaction (Saiki *et al.*, 1988). The PCR of the upstream region for Rv2531c and *phoPR* was done as described in the Materials and Methods (section 2.5). The PCR of MTCY159 and MTCY369 DNA were done using a number of different reaction parameters including varying the concentration of DNA, annealing temperature and primer concentration. In the most favourable conditions, reactions were heated at 94°C for 5 minutes for 1 cycle (hot start) in a thermal cycler. 0.5 µl of $Vent_R$ (exo-) DNA polymerase was added to each tube and amplification continued using the following thermal cycle parameters; denaturation for 1 minute at 95°C, annealing for 1 minute at 45°C, extension for 2 minutes at 72°C for a total of 34 cycles, followed by 1 minute at 95°C, 1 minute at 45°C and 10 minutes at 72°C for 1 cycle and holding the temperature at 4°C until the PCR tubes were removed and stored at –20°C. The annealing temperature was done at 52°C for amplification of P*pd*. The PCR products were visualised at approximately 301 bp and 1065 bp for the region upstream of Rv2531c and *phoPR* respectively, through a 1% (w/v) agarose gel (Figure 4.11). The 301 bp P*pd* and 1065 bp P*phoPR* bands were excised from the agarose gel and DNA was isolated using the Sephaglas Bandprep Kit.

4.4.4. Sequencing PCR products by automated sequencing.

The PCR products for the 301 bp P*pd* and 1065 bp P*phoPR* were sequenced using the ABI PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit using primers MTCY159-F/MTCY159-R and MTCY369-F/MTCY369-R respectively (see Material and Methods, Table 2.3). Sequence was analysed using AutoAssembler 1.4.0, Model 377 and the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

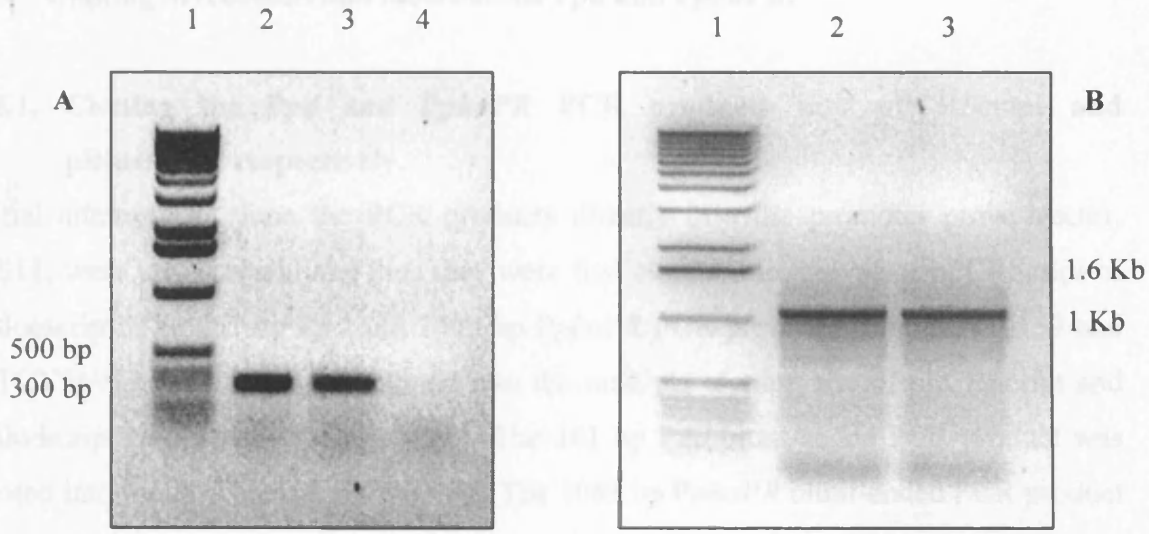


Figure 4.11. PCR to amplify the promoter for the Rv2531c and *phoPR* genes.
Amplification of the 5' region upstream of the genes Rv2531c and *phoPR* was done using primers MTCY159-F/MTCY159-R and MTCY369-F/MTCY369-R respectively. Lane 1 (photographs A and B) contains DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb) and in basepairs (bp). Photograph A, lanes 2 and 3 contains P*pd* PCR product at approximately 301 bp and lane 4 contains the negative control. Photograph B, lanes 2 and 3 contains P*phoPR* PCR product at approximately 1065 bp.

Sequence analysis confirmed that both the required regions upstream of Rv2531c and *phoPR* had been amplified (see Appendix 2).

4.5. Cloning *Mycobacterium tuberculosis* P*pd* and P*phoPR*.

4.5.1. Cloning the P*pd* and P*phoPR* PCR products into pPCRScript and pBluescript respectively.

Initial attempts to clone the PCR products directly into the promoter probe vector, pJS11, were unsuccessful and thus they were first cloned into the vector pPCRScript or pBluescript. The 301 bp P*pd* and 1065 bp P*phoPR* PCR products from MTCY159 and MTCY369 respectively, were cloned into the multiple cloning site of pPCRScript and pBluescript respectively (Figure 4.12). The 301 bp P*pd* blunt-ended PCR product was ligated into the *Srf* I site of pPCRScript. The 1065 bp P*phoPR* blunt-ended PCR product was ligated into pBluescript that had been digested with *Hind* III and subsequently dephosporylated to prevent recircularisation of the vector DNA. Each ligation product was introduced into *E. coli* strain DH5α by electroporation and transformants were selected on ampicillin, X-gal and IPTG. White colonies not expressing β-galactosidase were selected and plasmid DNA was prepared from these transformants as described in the Materials and Methods (section 2.3.1).

To confirm the presence of the 301 bp P*pd* DNA fragment in pPCRScript, plasmid DNA was digested with *Cla* I and *Hind* III. Restriction digestion with *Cla* I/*Hind* III resulted in two fragments of approximately 2961 bp and 301 bp; this recombinant plasmid was designated pJS14 (Figure 4.12). The 1065 bp P*phoPR* DNA fragment was digested with *Hind* III, which resulted in two fragments of approximately 2961 bp comprising of pBluescript and 1065 bp comprising of the cloned fragment, this recombinant plasmid was designated pJS15 (Figure 4.12). These experiments demonstrate successful cloning of the P*pd* and P*phoPR* fragments into pPCRScript and pBluescript respectively.

4.5.2. Subcloning the P*pd* and P*phoPR* fragment into the promoter probe vector, pJS11.

For subsequent subcloning of the DNA fragments of P*pd* and P*phoPR* into the promoter probe vector, pJS11, the plasmid pJS14 was digested with *Cla* I and *Hind* III

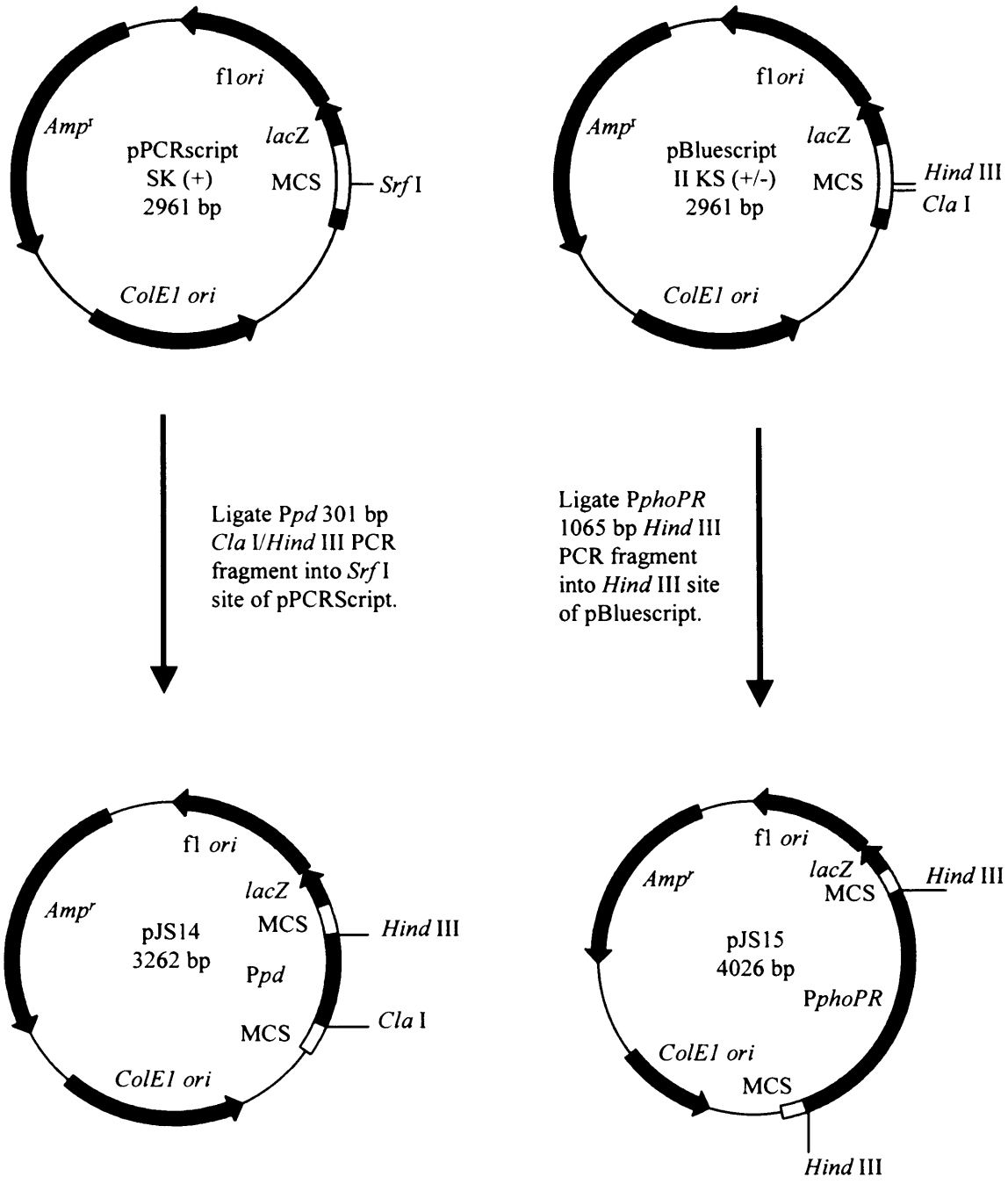


Figure 4.12. Cloning the P*pd* and P*phoPR* PCR products into pPCRScript and pBluescript respectively to construct pJS14 and pJS15.

The 301 bp P*pd* amplified fragment from MTCY159 was digested with C*la* I/*Hind* III and ligated into the C*la* I/*Hind* III site of pPCRScript to produce pJS14. The 1065 bp P*phoPR* amplified fragment from MTCY369 was digested with *Hind* III and ligated into the *Hind* III site of pBluescript to produce pJS15. Abbreviations are *Escherichia coli* origin of replication (*ColE1 ori*), ampicillin resistance (*Amp*^{r}) blue/white selection with β-galactosidase (*lacZ*), multiple cloning site (MCS) and f1 filamentous phage origin of replication (f1*ori*).

and pJS15 was digested with *Hind* III. The 301 bp and 1065 bp bands respectively were excised from the agarose gel and DNA fragments isolated. Plasmid DNA, pJS11, was partially digested with *Cla* I and subsequently digested with *Hind* III. The 301 bp P*pd* digested *Cla* I/*Hind* III fragment was ligated into the *Cla* I/*Hind* III digested sites of pJS11 and the 1065 bp P*phoPR Hind* III digested fragment was ligated into *Hind* III digested and dephosphorylated pJS11 (Figure 4.13., and Figure 4.14). The ligation products were introduced into *E. coli* strain DH5α by electroporation and transformants were selected on 200 μg/ml hygromycin B.

To confirm the presence of the 301 bp P*pd* DNA fragment in pJS11, DNA was digested with *Pvu* II. With one *Pvu* II site present in P*pd* and the second site in pJS11, restriction digest with *Pvu* II resulted in two fragments of approximately 5100 bp and 2500 bp. This recombinant plasmid was designated pJS12 (Figure 4.13., and Figure 4.15.A). The recombinant plasmid containing the 1065 bp P*phoPR* DNA fragment was also confirmed by restriction digestion with *Pvu* II. Restriction digestion with *Pvu* II resulted in two fragments of approximately 5500 bp and 2900 bp, with one *Pvu* II site present in P*phoPR*. This recombinant plasmid was designated pJS13 (Figure 4.14., and Figure 4.15.B). These digests demonstrated successful cloning of the P*pd* and P*phoPR* fragments into the promoter probe vector, pJS11.

4.5.3. Orientation of the P*phoPR* fragment in pJS13.

Given that only one restriction endonuclease was used in the cloning of the P*phoPR* DNA fragment into pJS11, the orientation of the insert was unknown. In order to identify the correct orientation of the promoter P*phoPR* with *lux*, the plasmid pJS13 was digested with *Cla* I. Two orientations were observed. One had P*phoPR* aligned in the opposite direction to the *luxAB* genes, thus resulting in 3 fragments of approximately 5300 bp, 2750 bp and 300 bp, which corresponded to the incorrect orientation. This recombinant plasmid was therefore renamed as pJS16 (Figures 4.16. and 4.17). The second orientation was the alignment of P*phoPR* in the same direction as the *luxAB* genes, thus resulting in 4 fragments of approximately 5300 bp, 2000 bp, 750 bp and 300 bp, which corresponded to the desired orientation. This recombinant plasmid was confirmed as pJS13 and was used for further work (Figure 4.16., and Figure 4.17).

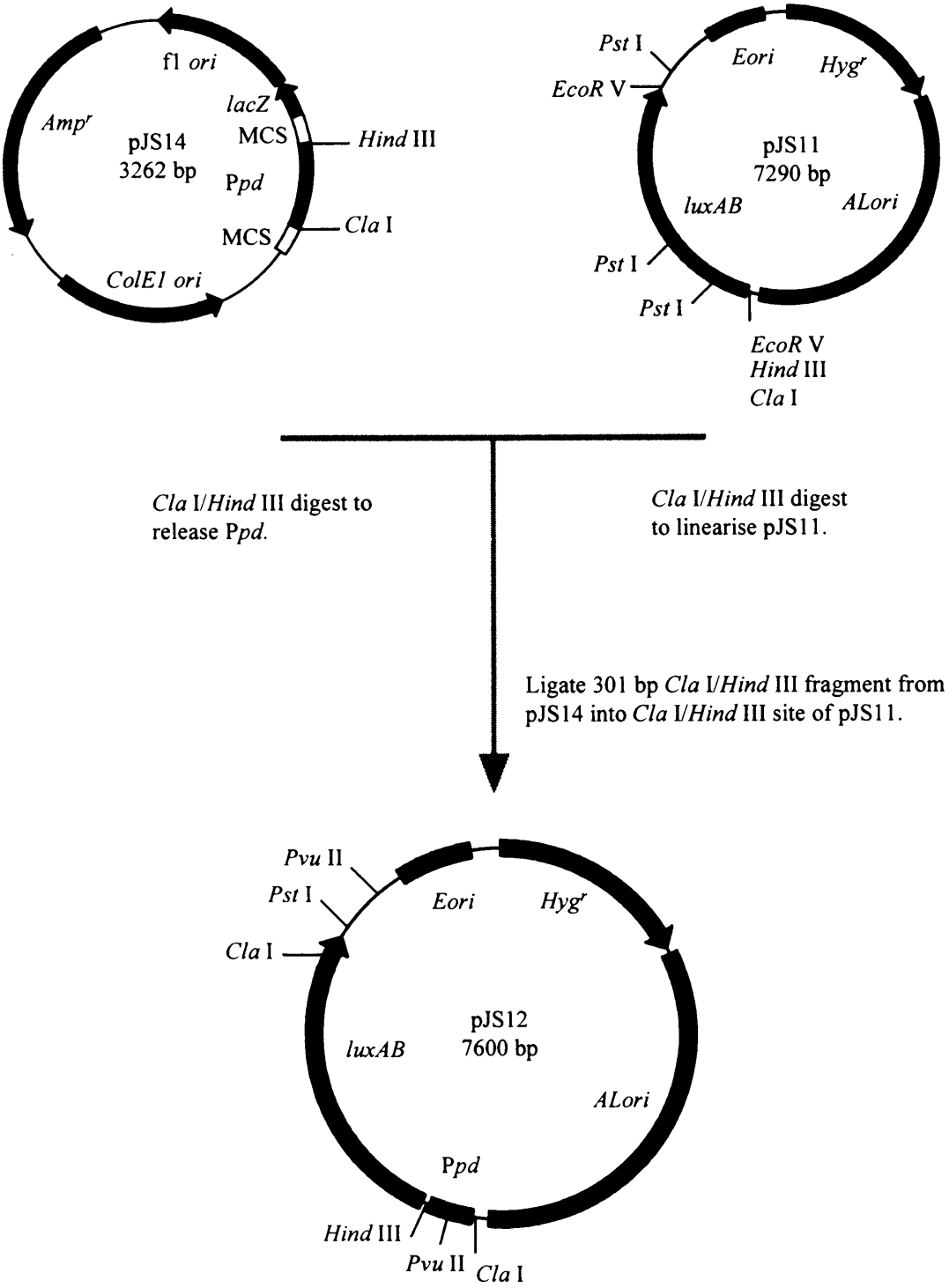


Figure 4.13. Subcloning the P*pd* fragment into the promoter probe vector, pJS11 to produce pJS12.

The plasmid pJS14 was digested with *Cla* I/*Hind* III to remove the 301 bp P*pd* fragment and was subsequently ligated to the *Cla* I/*Hind* III site of pJS11 to produce pJS12. Some of the abbreviations are as in Figure 4.12. Mycobacterial origin of replication (*ALori*), *Escherichia coli* origin of replication (*Eori*), hygromycin resistance (*Hyg*^{r}), and luciferase genes (*luxAB*).

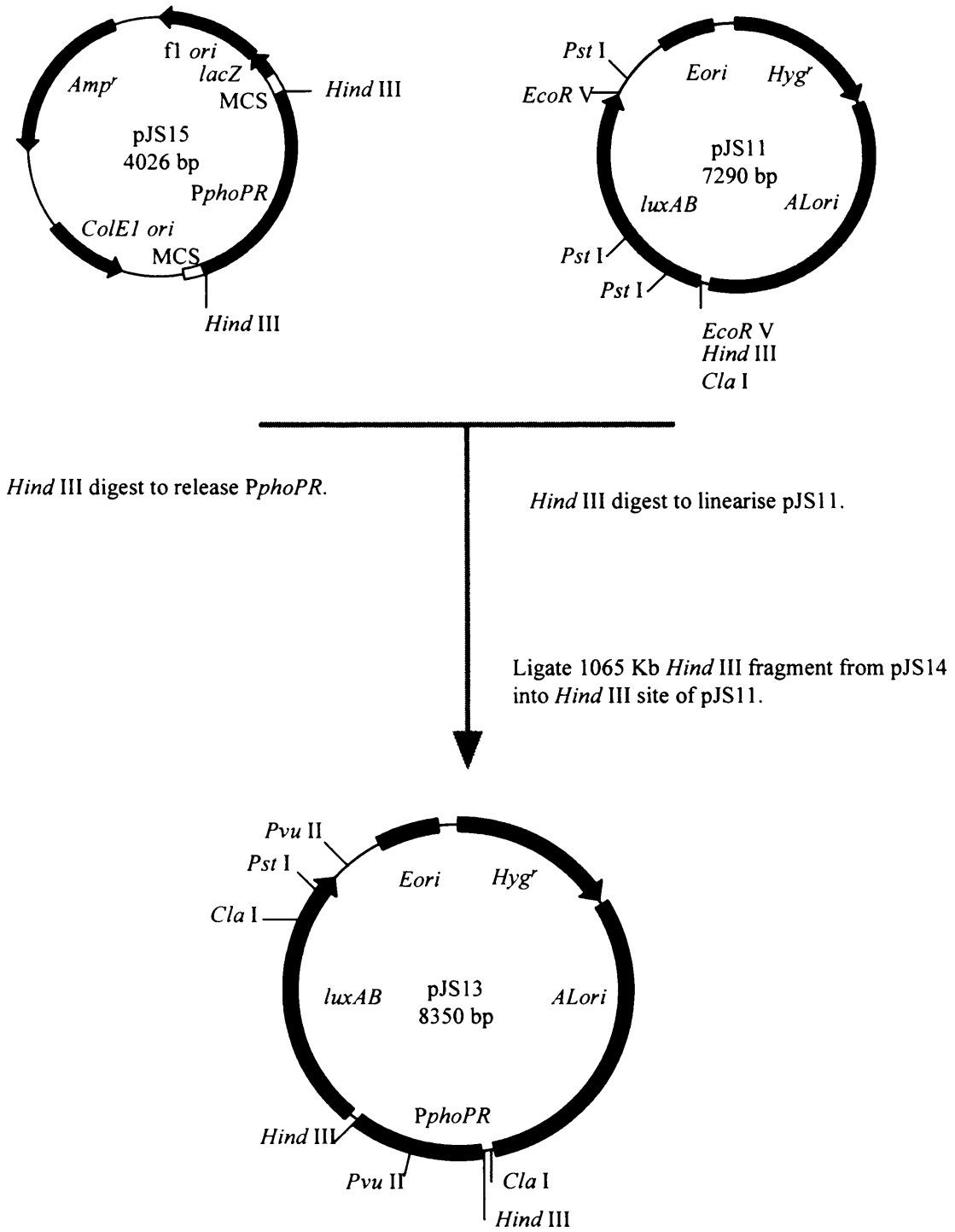


Figure 4.14. Subcloning the P*phoPR* fragment into the promoter probe vector, pJS11 to produce pJS13.

The plasmid pJS15 was digested with *Hind* III to remove the 1065 bp P*phoPR* fragment and was subsequently ligated to the *Hind* III site of pJS11 to produce pJS13. Some of the abbreviations are as in Figure 4.12. Mycobacterial origin of replication (*ALori*), *Escherichia coli* origin of replication (*Eori*), hygromycin resistance (*Hyg*^{r}), and luciferase genes (*luxAB*).

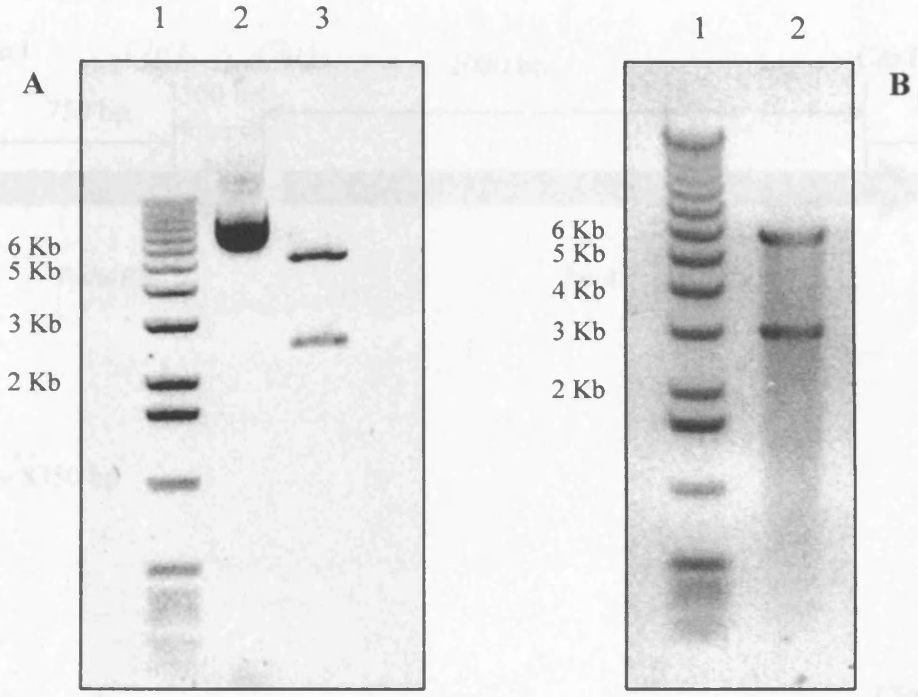


Figure 4.15. Subcloning the P*pd* and P*phoPR* fragment into the promoter probe vector, pJS11.

To confirm the presence of the 301 bp P*pd* DNA fragment and the 1065 bp P*phoPR* DNA fragment in pJS11, DNA was digested with *Pvu* II. Lane 1 (photographs A and B) contain DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb). Undigested P*pd* DNA (photograph A, lane 2). Restriction digestion of P*pd* with *Pvu* II resulted in two fragments of approximately 5100 bp and 2500 bp (photograph A, lane 3). Restriction digestion of P*phoPR* with *Pvu* II resulted in two fragments of approximately 5500 bp and 2900 bp (photograph B, lane 2).

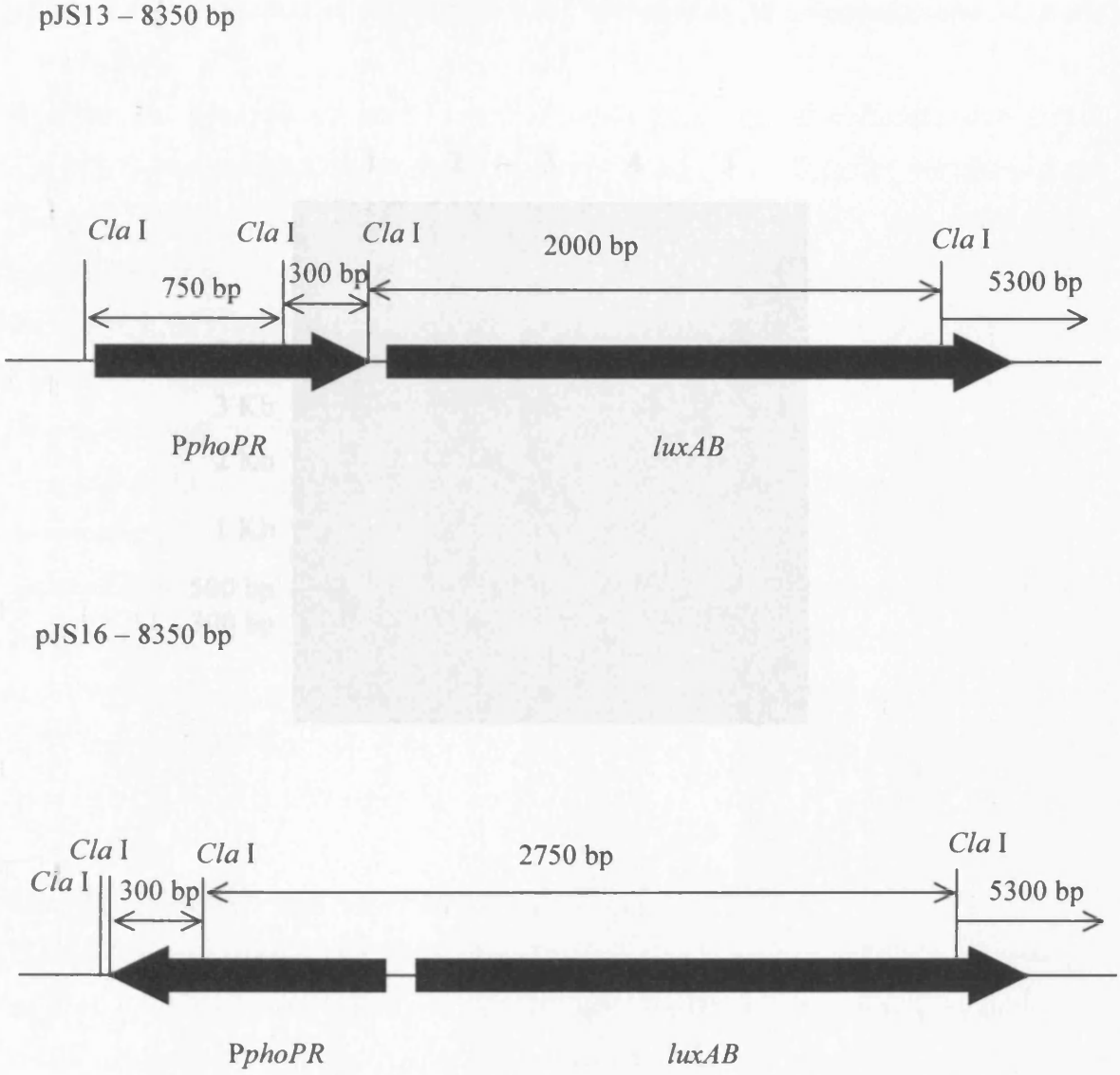


Figure 4.16. Orientation of the P*phoPR* fragment in pJS13.

The plasmid pJS13 was digested with *Cla* I and two orientations were observed. The desired orientation was the alignment of the P*phoPR* fragment in the same direction to that of the *luxAB* genes and this recombinant plasmid was confirmed as pJS13. The P*phoPR* fragment also aligned in the opposite direction to the *luxAB* genes, which corresponded to the incorrect orientation. This recombinant plasmid was therefore renamed and has been designated pJS16.

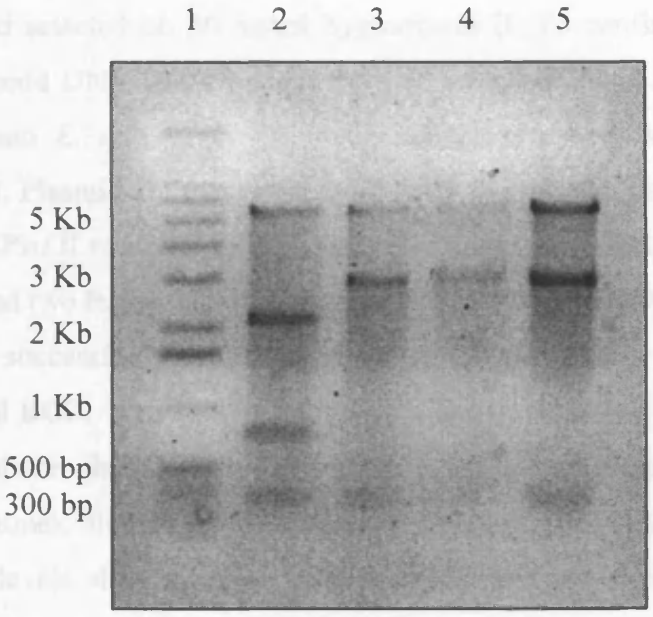


Figure 4.17. Orientation of the P*phoPR* fragment in pJS13.
The plasmid pJS13 was digested with *Cla* I and two orientations were observed. The incorrect orientation with the P*phoPR* fragment aligned in the opposite direction to the *luxAB* genes resulted in 3 fragments of approximately 5300 bp, 2750 bp and 300 bp (Lanes 3, 4 and 5) and was subsequently renamed pJS16. The desired orientation with the alignment of the P*phoPR* fragment in the same direction to that of the *luxAB* genes resulted in 4 fragments of approximately 5300 bp, 2000 bp, 750 bp and 300 bp and was designated pJS13. Lane 1 contain DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb) and basepairs (bp).

4.5.4. Transformation of pJS12 and pJS13 DNA into *M. smegmatis* and *M. bovis* BCG.

Mycobacterium smegmatis mc^2155 and *M. bovis* BCG were transformed with pJS12 and pJS13 and selected on 50 μg/ml hygromycin B. To confirm the presence of the plasmids, plasmid DNA was prepared from *M. smegmatis* and BCG and subsequently transformed into *E. coli* strain DH5α. Transformants were selected on 200 μg/ml hygromycin B. Plasmid DNA was prepared from these transformants and subsequently digested with *Pvu* II resulting in two fragments of approximately 5100 bp and 2500 bp from pJS12 and two fragments of approximately 5500 bp and 2900 bp from pJS13. This demonstrated successful transformation of pJS12 and pJS13 plasmid DNA into *M. smegmatis* and BCG. Transformed mycobacteria were checked for light production by adding decanal onto the lid of the Petri-dish and exposing the transformants in complete darkness to Cronex film for several minutes or longer. The X-ray films were processed and very low levels of light was detected from *M. smegmatis* (pJS12). Higher levels of light was detected from *M. smegmatis* (pJS13) (Figure 4.18). No light was detected from BCG (pJS12) or BCG (pJS13).

4.6. Mycobacteria and the acid tolerance response.

The existence of a regulatory system in *Salmonella typhimurium* enables it to survive at an even lower pH than its preferred growth pH, through adaptation to a moderate pH before exposure to an extreme acidity. This is called the acid tolerance response (ATR) (Foster and Hall, 1990; Foster, 1995). The inducible acid tolerance response of *S. typhimurium* involves two stages, the pre-acid shock when the adaptation of the organism to a relatively mild acidic external pH of 5.8 occurs (adaptive pH) and the second stage, the post-acid shock that occurs on further challenge at an extreme acid external pH of 3.3 (lethal pH). Hence, a theory of an ATR in *Mycobacterium* has arisen from the substantial evidence of these systems in *S. typhimurium* and *Escherichia coli.* In order to test the hypothesis as to whether two genes Rv2531c and *phoPR*, could be involved in acid tolerance in mycobacteria has been tested by observing the increased expression of the *lux* reporter system downstream of the promoters of these two genes. In addition, a knockout mutation of the Rv2531c gene was constructed in *Mycobacterium smegmatis* to observe whether this gene could be involved in acid tolerance. This recombinant was subsequently used to test for an acid tolerance

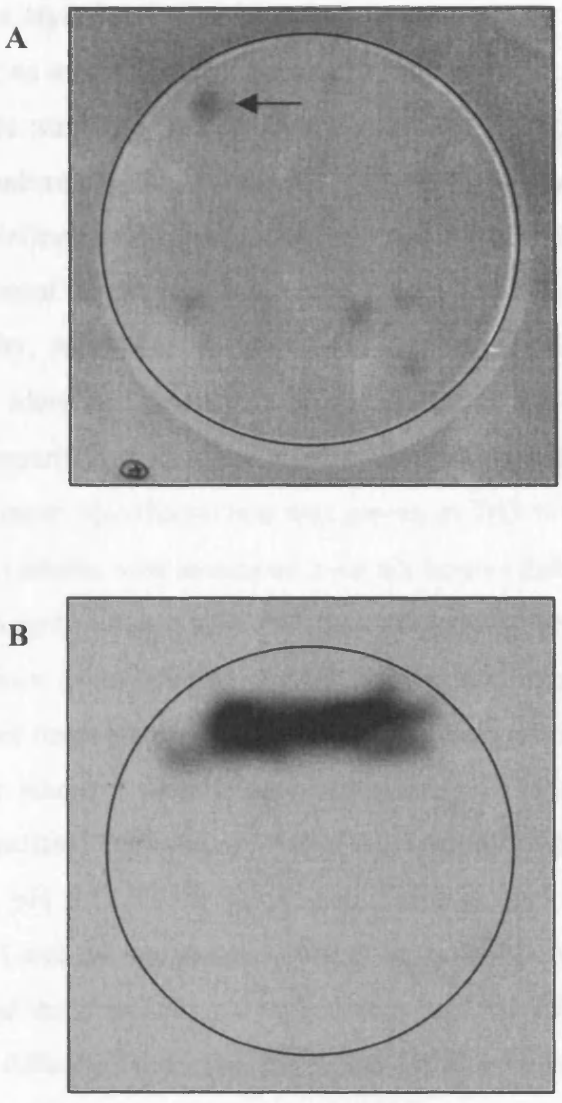


Figure 4.18. Transformation of pJS12 and pJS13 DNA into *M. smegmatis*.
M. smegmatis was transformed with pJS12 and pJS13 DNA and the transformants were exposed to decanal in complete darkness and Cronex film overlaid for several minutes or longer. Very low levels of light was detected from *M. smegmatis* (pJS12) (photograph A) and higher levels of light was detected from *M. smegmatis* (pJS13) (photograph B). (Image of two Petri-dishes).

response. The construction of a *Mycobacterium tuberculosis* Rv2531c gene knockout and testing for an acid tolerance response is discussed in Chapter 5.

4.6.1. Survival of *Mycobacterium smegmatis* over a range of pH.

Prior to testing for an acid tolerance response, survival of *M. smegmatis* was tested over a range of pH. This was done to identify the adaptive and lethal pH of the recombinant *M. smegmatis* transformed with either pJS12 or pJS13 for use in subsequent work. The adaptive pH was defined as the lowest external pH at which *M. smegmatis* continued to replicate and the lethal pH was defined as the lowest external pH that caused significant decrease in viability. Although the adaptive and lethal pH for wild-type *M. smegmatis* mc^2155 had been identified previously (Gordon, 1995; O'Brien, 1995, University of Leicester), these experiments were repeated to verify the results before further work was done. The recombinant *Mycobacterium* was grown in 7H9 broth at pH ranging from pH 7.6 to pH 2.5 and viability was measured over six hours (Table 4.3.a-d). The 7H9 broth was acidified with hydrochloric acid (HCl) or phosphoric acid (H_3PO_4). Two different acids were used since it has been suggested that the acidulant could result in variations in the acid tolerance response (Foster, 1999). Using the criteria that defined the adaptive and lethal pH, the adaptive pH for *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) using HCl was identified between pH 4.5 and pH 4.0 and the lethal pH for both strains was identified as pH 3.0 (Table 4.3.a. and Table 4.3.b.). The adaptive pH for *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) using H_3PO_4 was identified between pH 5.0 and pH 4.5 and the lethal pH for both strains was identified as pH 3.0 (Table 4.3.c and Table 4.3.d.). Although the lethal pH of 2.5 could have been chosen for both strains it was decided that pH 3.0 should be used instead since previous studies demonstrated that pH 2.5 would be to lethal even for the adapted bacteria. Two adaptive pHs were identified for both strains to ensure greater possibility of observing an ATR. It was interesting to see that *M. smegmatis* survived at pH 2.5 over six hours using HCl as the acidulant since previous studies demonstrated that bacteria exposed to pH 2.5 were rapidly killed (Gordon, 1995; O'Brien, 1995).

4.6.2. Testing *Mycobacterium smegmatis* for an acid tolerance response.

The adaptive and lethal pHs identified from the survival of *Mycobacterium smegmatis* over a range of pH were used to test for an acid tolerance response in *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13). An acid tolerance response was tested using

Experiment 1.				
Time (hrs)	**0**	**2**	**4**	**6**
pH				
7.6	7.28	7.49	7.39	7.67
6	7.18	7.52	7.68	7.91
5	7.18	7.26	7.39	7.50
4.5	7.18	7.26	7.41	7.49
4	7.19	7.19	7.40	7.52
3.5	7.05	7.11	7.10	6.95
3	7.14	6.74	6.53	6.15
2.5	7.11	6.47	5.88	5.21

Experiment 2.				
Time (hrs)	**0**	**2**	**4**	**6**
pH				
7.6	7.19	7.34	7.58	7.74
6	7.12	7.35	7.56	7.82
5	7.18	7.25	7.33	7.63
4.5	7.13	7.23	7.33	7.45
4	7.12	7.29	7.43	7.48
3.5	7.15	7.16	7.09	6.95
3	7.04	6.85	6.53	6.18
2.5	7.13	6.64	6.13	5.44

Table 4.3.a. Survival of *Mycobacterium smegmatis* (pJS12) over a range of pH.
Results show the log colony forming units/ml for *M. smegmatis* (pJS12) over 6 hours after incubation at each pH. Cultures were acidified with hydrochloric acid (HCl). All values are the mean of two counts taken from a single sample at the appropriate time point.

Experiment 1.

Time (hrs)	0	2	4	6
pH				
7.6	6.90	7.13	-	7.37
6	6.84	7.12	-	7.56
5	6.74	7.01	-	7.19
4.5	6.88	7.05	-	7.21
4	6.77	7.03	-	6.83
3.5	6.82	6.70	-	6.32
3	6.85	6.26	-	5.06
2.5	6.81	6.08	4.78	4.37

Experiment 2.

Time (hrs)	0	2	4	6
pH				
7.6	6.84	6.90	7.29	7.06
6	6.85	7.16	7.42	7.40
5	6.86	6.77	7.05	7.32
4.5	6.76	7.12	7.08	7.00
4	6.79	6.68	6.91	6.85
3.5	6.89	6.64	6.48	6.19
3	6.82	6.14	5.52	4.93
2.5	6.80	6.11	5.35	4.30

Table 4.3.b. Survival of *Mycobacterium smegmatis* (pJS13) over a range of pH.
Results show the log colony forming units/ml for *M. smegmatis* (pJS13) over 6 hours after incubation at each pH. Cultures were acidified with hydrochloric acid (HCl). All values are the mean of two counts taken from a single sample at the appropriate time point (- represents no data).

Experiment 1.

Time (hrs)	0	2	4	6
pH				
7.6	7.11	7.11	7.38	7.51
6	7.17	7.10	7.19	7.50
5	7.11	7.13	7.12	7.26
4.5	7.14	7.10	7.21	7.09
4	6.99	6.88	6.96	6.72
3.5	7.12	6.42	5.99	5.29
3	7.22	6.07	5.05	4.28
2.5	6.94	5.30	3.00	2.60

Experiment 2.

Time (hrs)	0	2	4	6
pH				
7.6	7.03	7.17	7.38	7.53
6	7.14	7.07	7.58	7.26
5	7.09	7.07	7.24	7.18
4.5	7.03	7.12	7.23	7.26
4	7.17	6.90	6.76	6.69
3.5	7.20	6.36	5.90	5.30
3	6.97	6.06	5.86	4.21
2.5	7.21	5.30	3.28	2.30

Table 4.3.c. Survival of *Mycobacterium smegmatis* (pJS12) over a range of pH.
Results show the log colony forming units/ml for *M. smegmatis* (pJS12) over 6 hours after incubation at each pH. Cultures were acidified with phosphoric acid (H_3PO_4). All values are the mean of two counts taken from a single sample at the appropriate time point.

Experiment 1.

Time (hrs)	0	2	4	6
pH				
7.6	7.05	7.38	7.47	7.53
6	7.10	7.20	7.43	7.49
5	7.12	7.28	7.34	7.28
4.5	7.05	7.27	7.33	7.18
4	7.10	7.07	7.07	7.80
3.5	7.17	6.35	5.95	5.73
3	7.21	5.63	4.54	4.19
2.5	7.23	4.92	3.64	2.30

Experiment 2.

Time (hrs)	0	2	4	6
pH				
7.6	7.13	7.25	7.37	7.43
6	7.15	7.35	7.45	7.59
5	7.11	7.21	7.28	7.22
4.5	6.91	7.25	7.24	7.19
4	7.12	7.14	6.62	6.93
3.5	7.16	6.35	6.07	5.68
3	7.19	5.58	4.28	4.20
2.5	7.14	5.16	3.63	2.70

Table 4.3.d. Survival of *Mycobacterium smegmatis* (pJS13) over a range of pH.
Results show the log colony forming units/ml for *M. smegmatis* (pJS13) over 6 hours after incubation at each pH. Cultures were acidified with phosphoric acid (H_3PO_4). All values are the mean of two counts taken from a single sample at the appropriate time point.

hydrochloric acid (HCl) and phosphoric acid (H_3PO_4) for each recombinant *M. smegmatis*. Initially, *M. smegmatis* (pJS12) was grown in 7H9 broth at a control pH of 7.6 and adapted at a pH of 5.0 or pH 4.5 using H_3PO_4 and incubated to allow one doubling before exposing cultures at a lethal pH of 3.0. Samples of each culture were removed at 0, 1, 2, 3, and 4 hours and viable counts were done for the unadapted and adapted cultures. Initial experiments with *M. smegmatis* (pJS12) using H_3PO_4 to acidify the medium failed to exhibit an ATR over a four hour period (Figure 4.19). For example, percentage viability at the one hour time point was 12% for cultures adapted at pH 5.0, 93% for cultures adapted at pH 4.5 and 125% for unadapted cultures at pH 7.6. Thus, under these conditions *M. smegmatis* (pJS12) did not demonstrate an ATR.

However, since an ATR was not exhibited with *M. smegmatis* (pJS12) when tested over four hours, the experiment was repeated using *M. smegmatis* (pJS13) but this time viability at pH 3.0 was measured over a period of six hours (Figure 4.20). Cultures may have required a longer time period before an ATR was observable. When the adapted and unadapted cultures were compared at each time point, cultures adapted at a pH of 5.0 survived significantly ($P< 0.05$) better at pH 3.0 only at the three hour time point. Percentage viability at the three hour time point was 5% for cultures adapted at pH 5.0 and 3% for unadapted cultures. Also, cultures adapted at a pH of 4.5 survived significantly ($P< 0.05$) better at pH 3.0 at the three hour and four hour time points compared to the unadapted cultures. Thus, under these conditions *M. smegmatis* (pJS13) did demonstrate an ATR when H_3PO_4 was the acidulant. However, the ATR was found to be temporary since cultures adapted at a pH of 5.0 or pH 4.5 did not survive better at pH 3.0 at the six hour time point compared to the unadapted cultures.

Because a temporary ATR had been observed with *M. smegmatis* (pJS13) and also because *M. smegmatis* (pJS12) failed to exhibit an ATR, it was subsequently decided to measure the survival of the strains at a lethal pH of 2.5 over six hours. *M. smegmatis* (pJS13) was adapted at a pH of 5.0 or pH 4.5 and exposed to a lethal pH of 2.5 with H_3PO_4 and viability was measured over 6 hours (Figure 4.21). Cultures adapted at a pH of 5.0 or pH 4.5 survived significantly ($P< 0.01$) better at pH 2.5 compared to the unadapted cultures at all time points. For example, percentage viability at the one hour

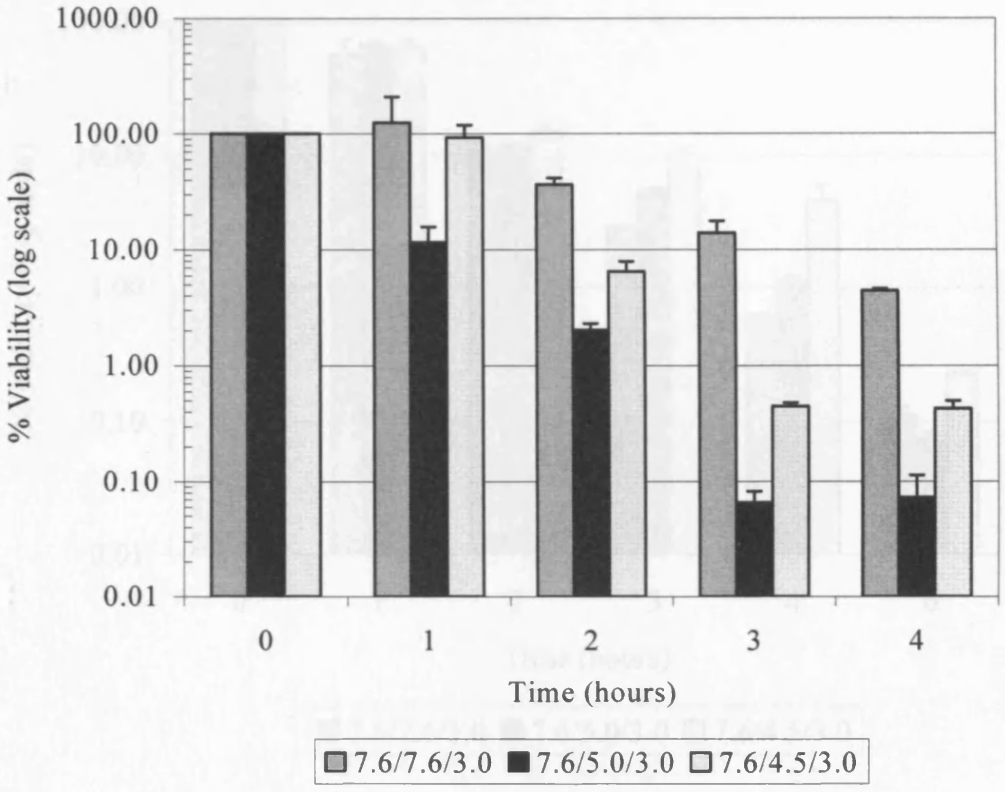


Figure 4.19. Testing *M. smegmatis* (pJS12) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 for two hours before exposure at a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS12) over 4 hours. All values represent the mean ± S.E.M. of two experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.0 or 4.5 at 0 time are 7.47, 8.36 and 7.74, respectively.

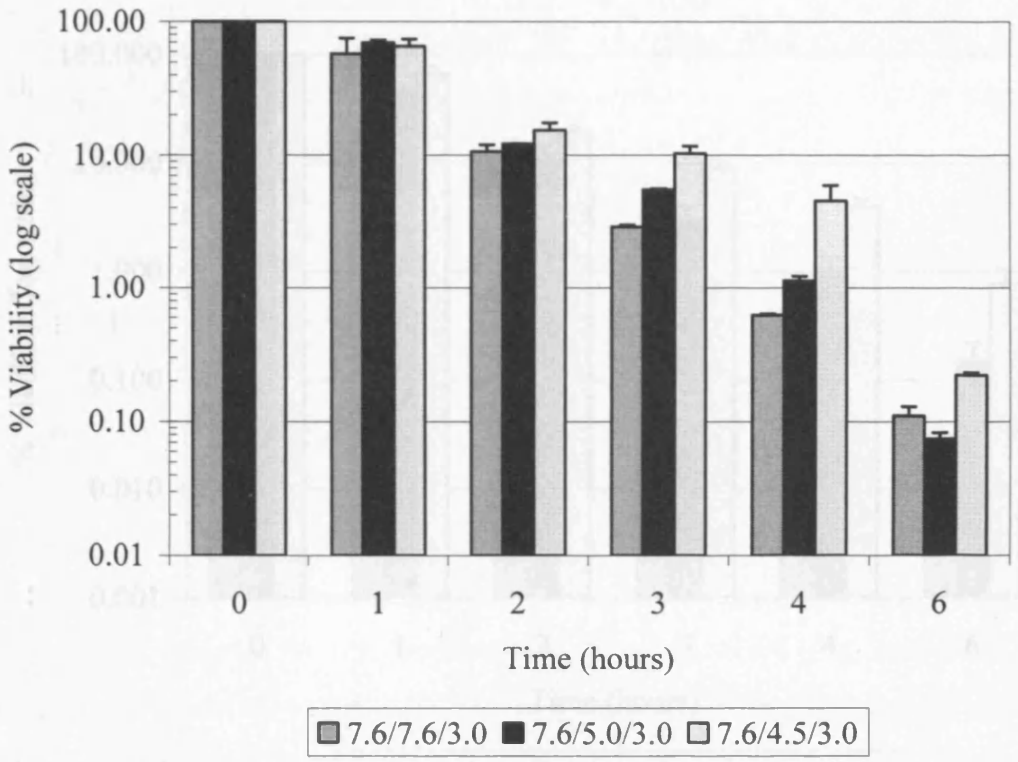


Figure 4.20. Testing *M. smegmatis* (pJS13) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 (unadapted) for two hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS13) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.0 or 4.5 at 0 time are 8.37, 8.36 and 8.29, respectively.

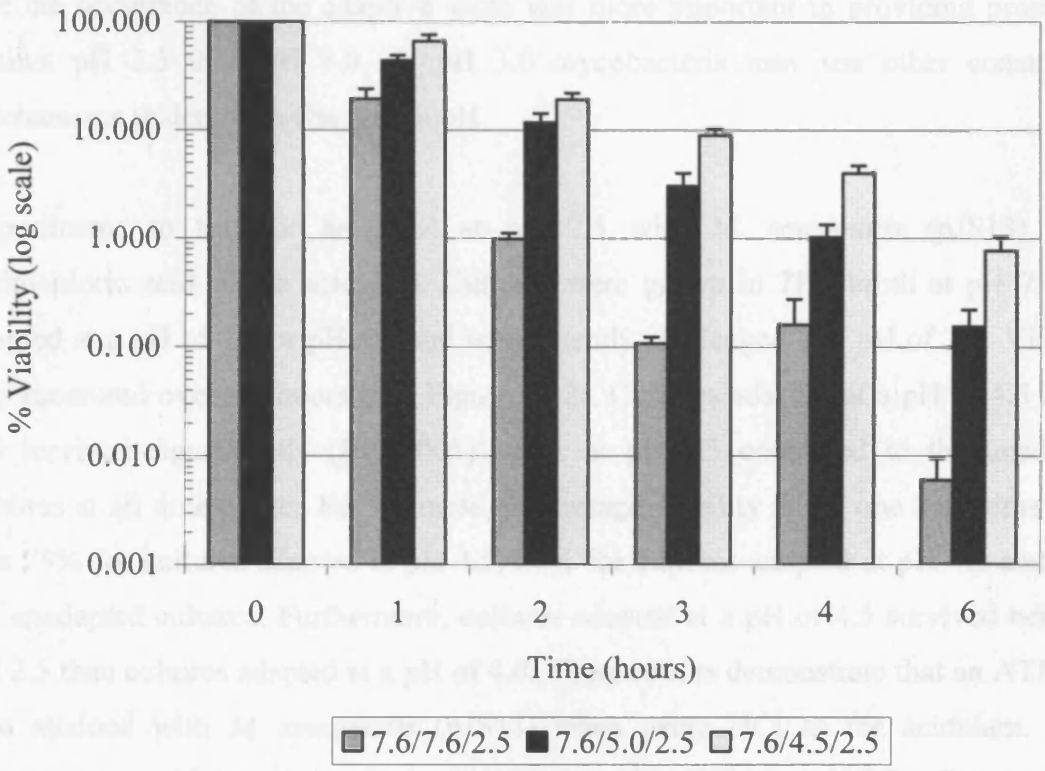


Figure 4.21. Testing *M. smegmatis* (pJS13) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 for two hours before exposure at a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS13) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.0 or 4.5 at 0 time are 8.30, 8.21 and 8.08, respectively.

time point was 44% for cultures adapted at pH 5.0, 66% for cultures adapted at pH 4.5 and 19% for unadapted cultures. Furthermore, cultures adapted at pH 4.5 survived significantly ($P< 0.001$) better at pH 2.5 than cultures adapted at pH 5.0 ($P< 0.01$). Under these conditions, *M. smegmatis* (pJS13) demonstrated a successful ATR when H_3PO_4 was the acidulant. *M. smegmatis* (pJS13) exposed at a lethal pH of 2.5 enabled the ATR to become more observable over time than exposure at pH 3.0. This suggested that the occurrence of the adaptive stage was more important in providing protection against pH 2.5 than pH 3.0. At pH 3.0 mycobacteria may use other constitutive mechanisms to deal with changes in pH.

Experiments to test for an ATR at pH 2.5 with *M. smegmatis* (pJS13) using hydrochloric acid as the acidulant. Cultures were grown in 7H9 broth at pH 7.6 and adapted at a pH of 4.5 or pH 4.0 and subsequently challenged at a pH of 2.5. Viability was measured over six hours (and Figure 4.22). Cultures adapted at a pH of 4.5 or pH 4.0 survived significantly ($P< 0.005$) better at pH 2.5 compared to the unadapted cultures at all time points. For example, percentage viability at the one hour time point was 79% for cultures adapted at pH 4.5, 80% for cultures adapted at pH 4.0 and 23% for unadapted cultures. Furthermore, cultures adapted at a pH of 4.5 survived better at pH 2.5 than cultures adapted at a pH of 4.0. These results demonstrate that an ATR was also attained with *M. smegmatis* (pJS13) when using HCl as the acidulant. Also, cultures appeared to survive better at pH 2.5 with HCl than H_3PO_4. For example, percentage viability at the three hour time point was 39% for cultures adapted at pH 4.5 using HCl as the acidulant and 9% for cultures adapted at pH 4.5 using H_3PO_4 as the acidulant. Statistically, a significant ($P< 0.05$) difference was seen between HCl and H_3PO_4 with *M. smegmatis* (pJS13) adapted at pH 4.5.

As reported on page 211 *M. smegmatis* (pJS12) did not exhibit an ATR when cultures were exposed at pH 3.0 following adaptation at a pH of 5.0 or 4.5 using H_3PO_4. Since an ATR had been seen with *M. smegmatis* (pJS13) tested at pH 2.5 but not at pH 3.0, *M. smegmatis* (pJS12) also was tested at a lethal pH of 2.5 over six hours (Figure 4.23). Cultures adapted at a pH of 5.0 or pH 4.5 survived significantly ($P< 0.005$) better at pH 2.5 compared to the unadapted cultures at all time points. For example, percentage viability at the one hour time point was 74% for cultures adapted at pH 5.0, 84% for cultures adapted at pH 4.5 and 29% for unadapted cultures at pH 7.6.

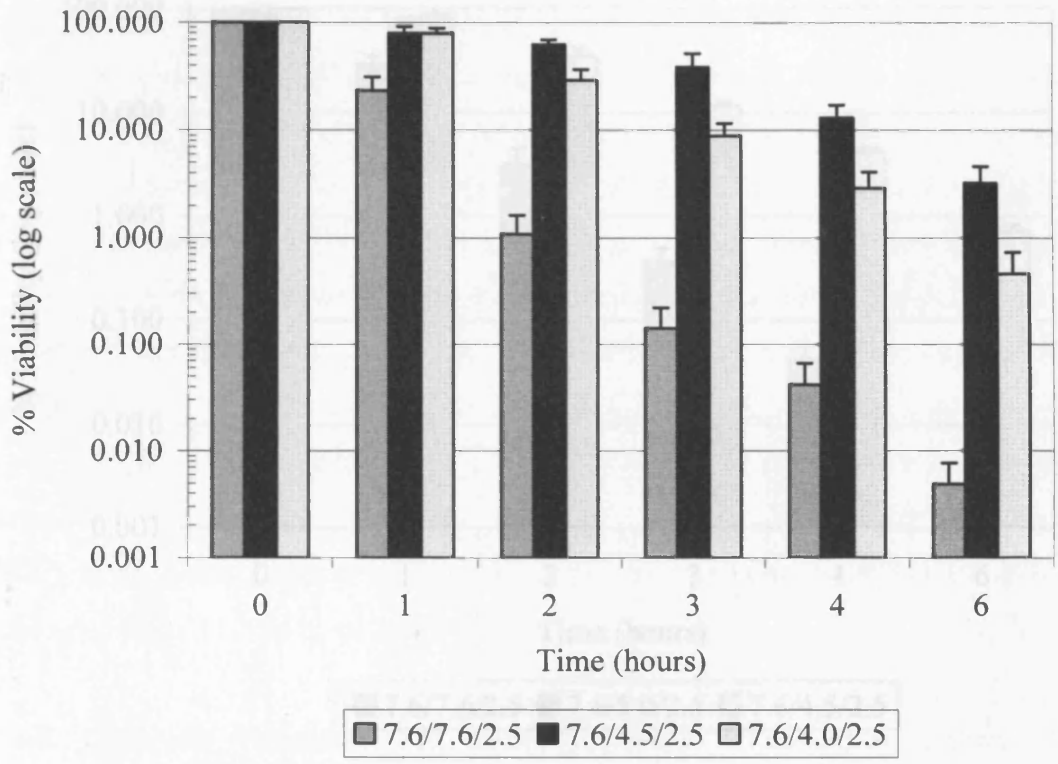


Figure 4.22. Testing *M. smegmatis* (pJS13) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or pH 4.0 for four hours or grown at pH 7.6 for two hours before exposure at a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS13) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 4.5 and 4.0 at 0 time are 8.27, 8.21 and 8.02, respectively.

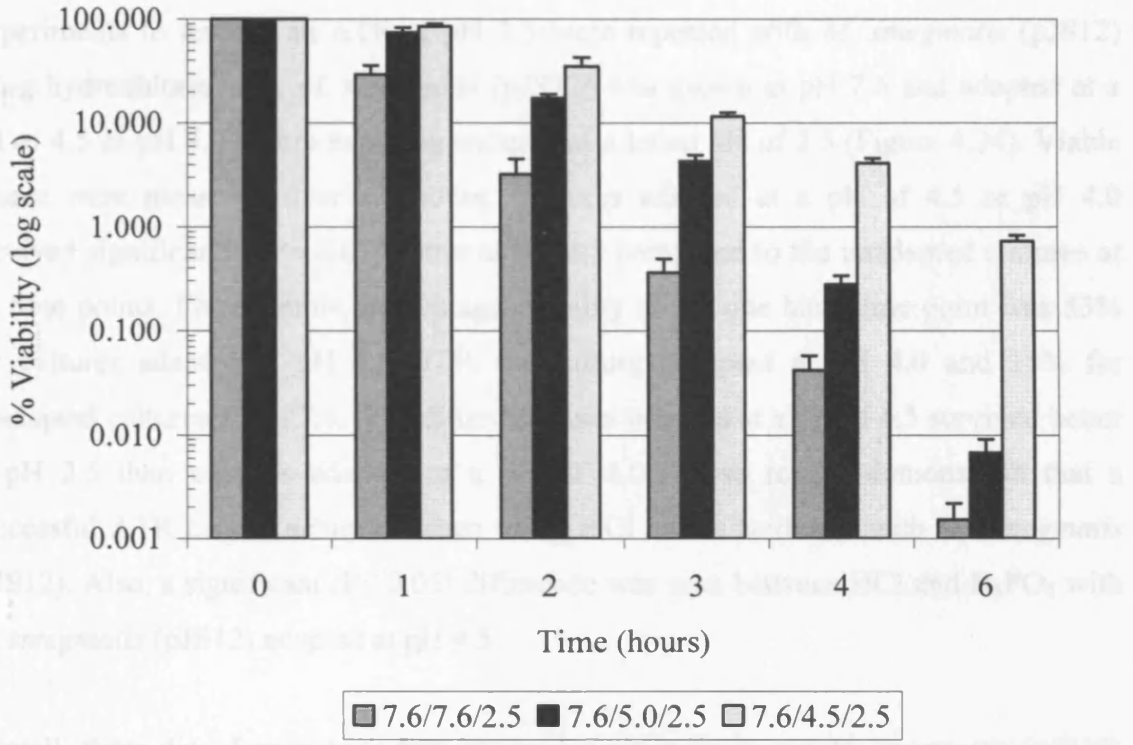


Figure 4.23. Testing *M. smegmatis* (pJS12) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 for two hours before exposure to a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS12) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.0 or 4.5 at 0 time are 8.34, 8.24 and 8.29, respectively.

In addition, cultures adapted at pH 4.5 survived significantly ($P< 0.001$) better at pH 2.5 than cultures adapted at pH 5.0 ($P< 0.005$). These results demonstrate that *M. smegmatis* (pJS12) was able to achieve a successful ATR and survive at a lethal pH of 2.5. Thus, the adaptive stage provided protection against the lethal pH of 2.5 but was not seen at a lethal pH of 3.0 when using H_3PO_4 as the acidulant.

Experiments to test for an ATR at pH 2.5 were repeated with *M. smegmatis* (pJS12) using hydrochloric acid. *M. smegmatis* (pJS12) was grown at pH 7.6 and adapted at a pH of 4.5 or pH 4.0 before exposing cultures at a lethal pH of 2.5 (Figure 4.24). Viable counts were measured over six hours. Cultures adapted at a pH of 4.5 or pH 4.0 survived significantly ($P< 0.01$) better at pH 2.5 compared to the unadapted cultures at all time points. For example, percentage viability at the one hour time point was 83% for cultures adapted at pH 4.5, 71% for cultures adapted at pH 4.0 and 15% for unadapted cultures at pH 7.6. In addition, cultures adapted at a pH of 4.5 survived better at pH 2.5 than cultures adapted at a pH of 4.0. These results demonstrate that a successful ATR can be achieved when using HCl as the acidulant with *M. smegmatis* (pJS12). Also, a significant ($P< 0.05$) difference was seen between HCl and H_3PO_4 with *M. smegmatis* (pJS12) adapted at pH 4.5.

Overall, these data demonstrate that *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) do possess an acid tolerance response. Both these strains have enhanced survival at a lethal pH of 2.5 provided they have prior adaptation at a moderate pH. This acid tolerance was seen when two types of acidulant are used, hydrochloric acid and phosphoric acid.

4.6.3. Testing for expression of *lux* in *M. smegmatis* at the 'adaptive' pH.

To determine whether the promoters for the two genes Rv2531c and *phoPR* were acid-inducible, the two strains *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) were exposed to acidic external pH and observed for increased expression of the *luxAB* genes downstream of the promoters P*pd* and P*phoPR*. The acidic external pH used for the following experiments were the adaptive pHs identified previously for each strain (see section 4.6.1). Each strain was tested with hydrochloric acid (HCl) and phosphoric acid (H_3PO_4) to observe any differences in expression.

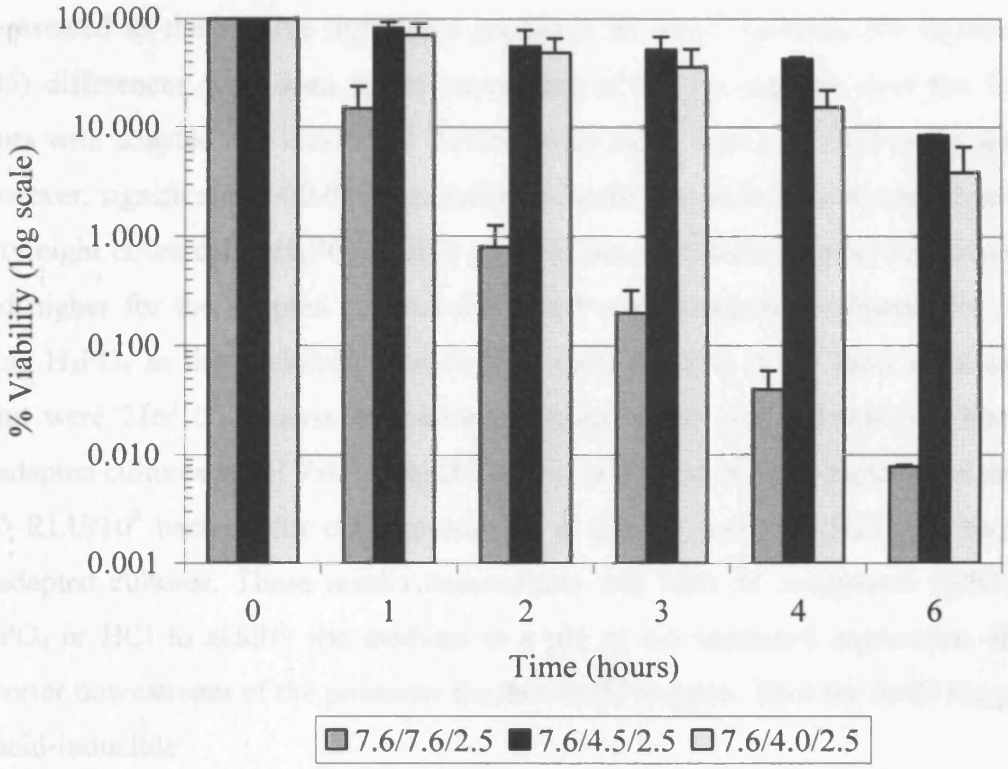


Figure 4.24. Testing *M. smegmatis* (pJS12) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or pH 4.0 for four hours or grown at pH 7.6 for two hours before exposure at a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS12) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 4.5 or 4.0 at 0 time are 8.19, 7.72 and 7.37, respectively.

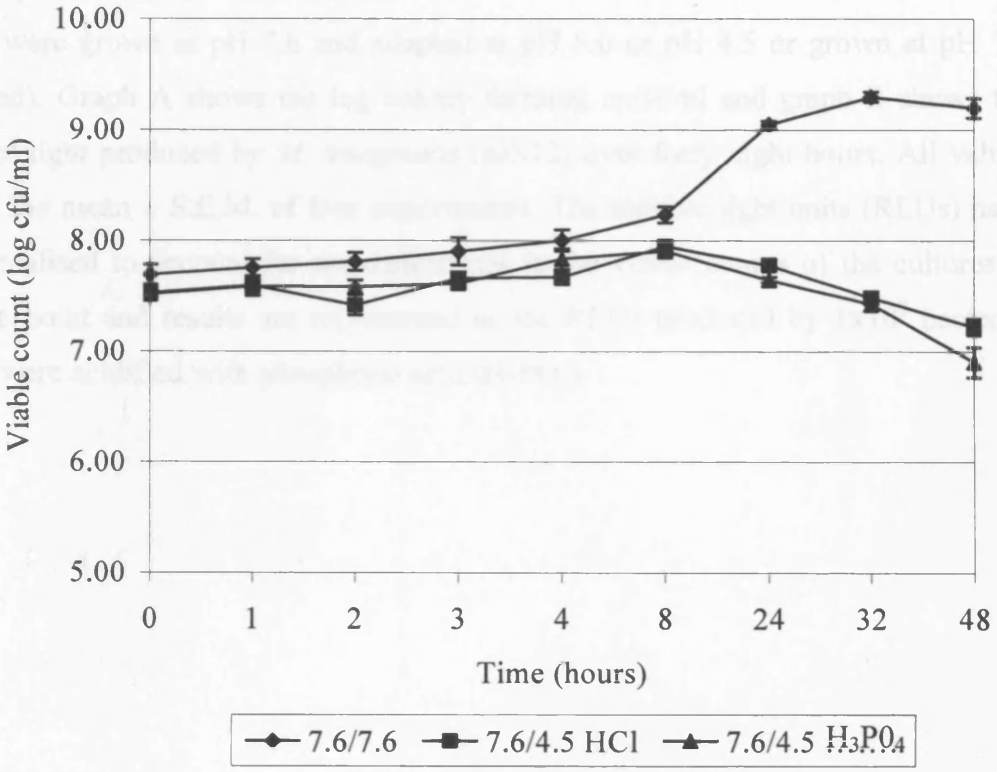
M. smegmatis (pJS12) was initially grown in 7H9 broth at a pH of 7.6 and subsequently exposed to a pH of 4.5 or placed into fresh medium at the control pH of 7.6 (Figure 4.25). The medium was acidified with either H_3PO_4 or HCl. Samples of each culture were immediately removed at 0, 1, 2, 3, 4, 8, 24, 32 and 48 hours and bioluminescence was measured. In addition, viable counts were done for the unadapted (pH 7.6) and adapted (pH 4.5) cultures. In order to normalise the amount of light produced to account for the differences in the viable counts at each time point, the results have been represented as the relative light units produced by $1x10^8$ bacteria. No significant ($P> 0.05$) differences were seen in the expression of the *lux* reporter over the first eight hours with adapted and unadapted cultures with either H_3PO_4 or HCl as the acidulants. However, significant ($P< 0.01$) expression of *luxAB* was induced at twenty four hours to forty eight hours using H_3PO_4 or HCl as acidulant. Bioluminescence was around 5000-fold higher for the adapted cultures compared with unadapted cultures. For example, using H_3PO_4 as the acidulant, relative light units (RLUs) at the forty eight hour time point were $216/10^8$ bacteria for cultures adapted at pH 4.5 and $0.04/10^8$ bacteria for unadapted cultures at pH 7.6. Using HCl as the acidulant, at forty eight hours there were 190 $RLU/10^8$ bacteria for cultures adapted at pH 4.5 and 0.04 $RLU/10^8$ bacteria for unadapted cultures. These results demonstrate that with *M. smegmatis* (pJS12) using H_3PO_4 or HCl to acidify the medium at a pH of 4.5 increased expression of the *lux* reporter downstream of the promoter for the Rv2531c gene. Thus the Rv2531c promoter is acid-inducible.

To determine whether the promoter for the gene *phoPR* was also acid-inducible, *M. smegmatis* (pJS13) was grown in 7H9 broth at a pH of 7.6 and subsequently exposed to pH 5.0 or pH 4.5 or placed into fresh medium at a control pH of 7.6. The medium was acidified with H_3PO_4. Samples of each culture were removed at time points over 48 hours and bioluminescence immediately measured. Viable counts were also done for the unadapted (pH 7.6) and adapted (pH 5.0 or pH 4.5) cultures (Figure 4.26). Cultures exposed at pH 5.0 or pH 4.5 appeared to show relatively small increases in bioluminescence early in the experiment but continued to increase gradually over eight hours compared with unadapted cultures (Figure 4.26). Statistically these levels were significantly ($P< 0.05$) different between adapted cultures at pH 5.0 or pH 4.5 compared with unadapted cultures from two hours to eight hours. After eight hours, bioluminescence appeared to increase considerably. For example, relative light units

Figure 4.25. Testing *M. smegmatis* (pJS12) for expression of *lux* at the adaptive pH with hydrochloric acid or phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or grown at pH 7.6 (unadapted). Graph A shows the log colony forming units/ml and graph B shows the amount of light produced by *M. smegmatis* (pJS12) over forty eight hours. All values represent the mean ± S.E.M. of four experiments. The relative light units (RLUs) have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the RLUs produced by $1x10^8$ bacteria. Cultures were acidified with either hydrochloric acid (HCl) or phosphoric acid (H_3P0_4).

A


10.00
9.00
8.00
7.00
6.00
5.00
Viable count (log cfu/ml)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/4.5 HCl
7.6/4.5 H3P04

B

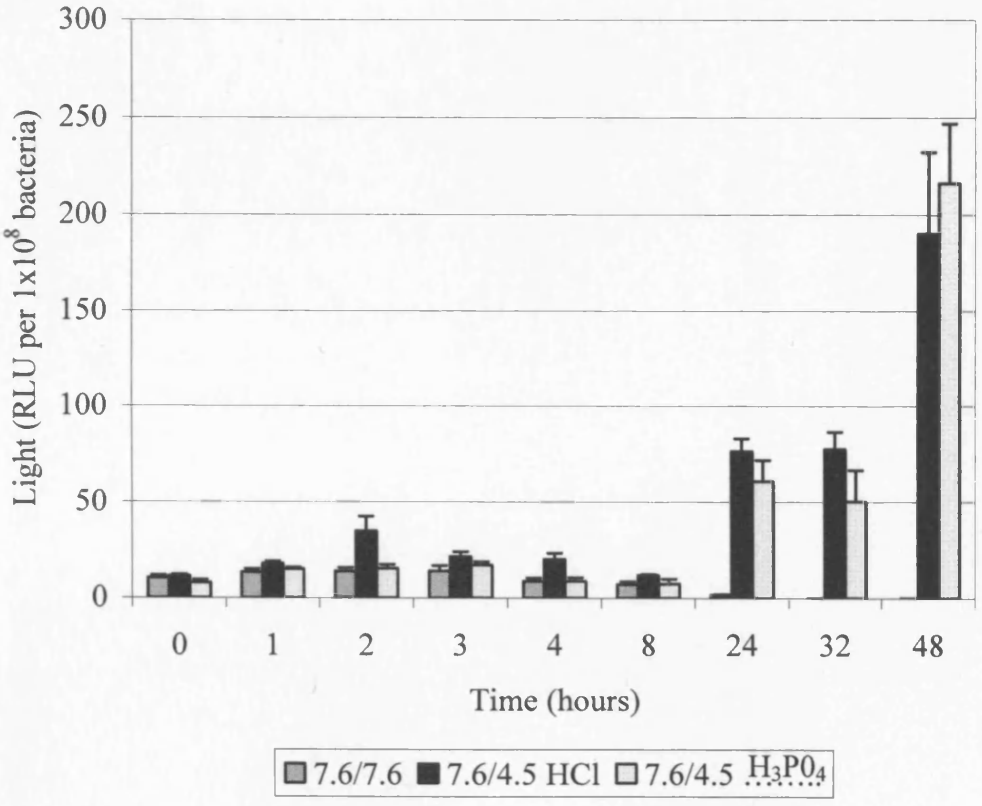
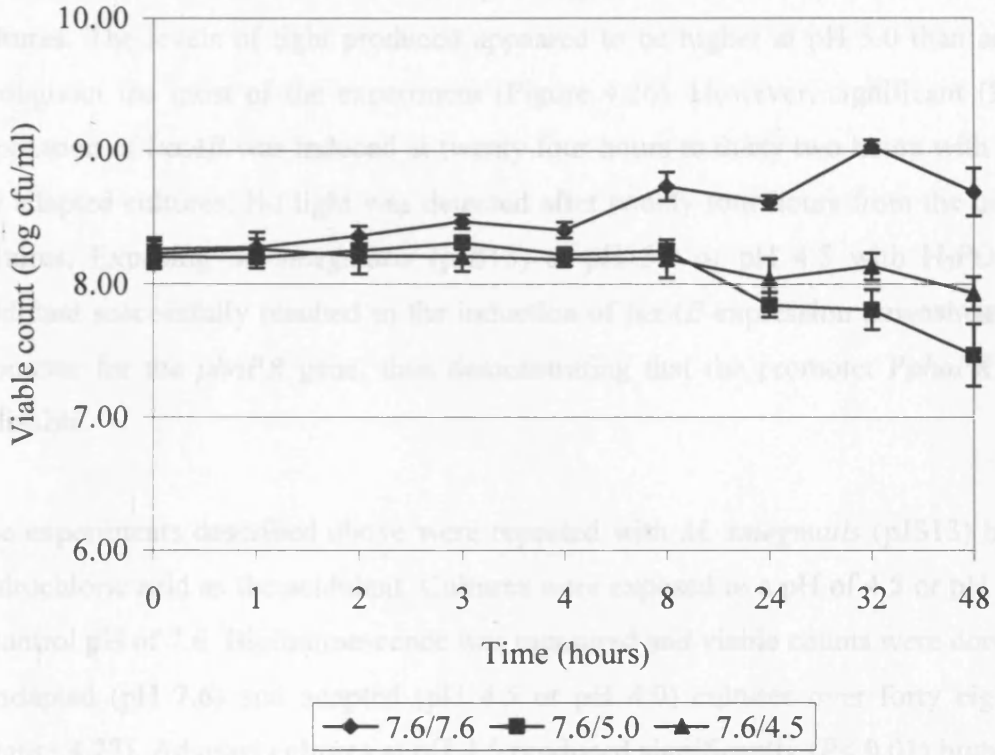

300
250
200
150
100
50
0
Light (RLU per 1x10^8 bacteria)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/4.5 HCl
7.6/4.5 H3P04

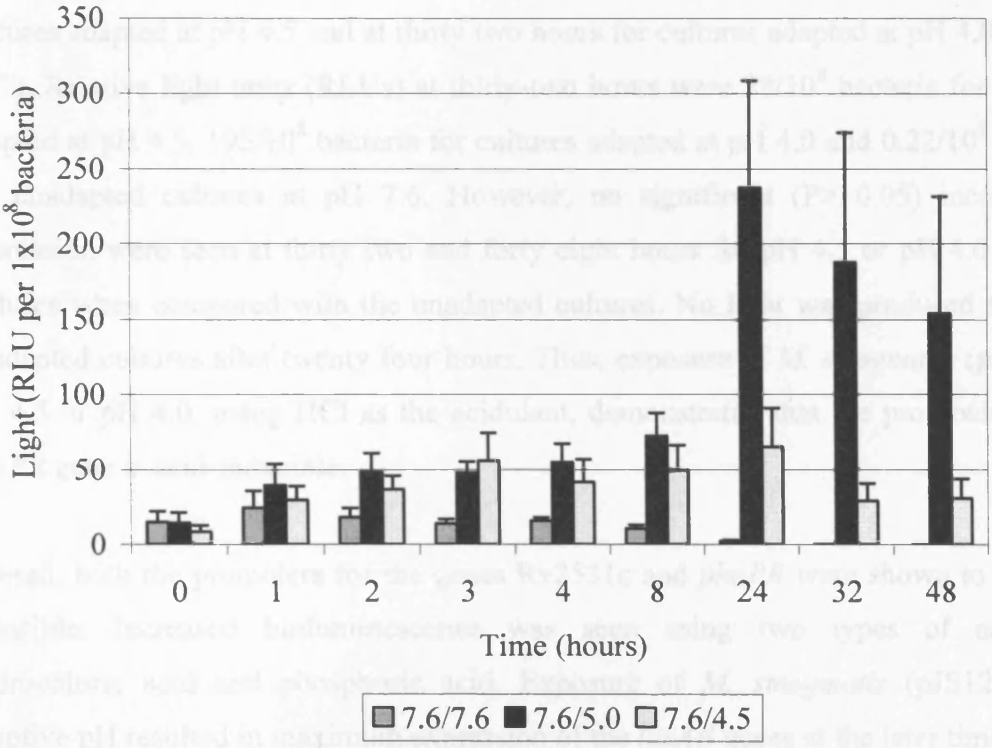
Figure 4.26. Testing *M. smegmatis* (pJS13) for expression of *lux* at the adaptive pH with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 or grown at pH 7.6 (unadapted). Graph A shows the log colony forming units/ml and graph B shows the amount of light produced by *M. smegmatis* (pJS13) over forty eight hours. All values represent the mean ± S.E.M. of four experiments. The relative light units (RLUs) have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the RLUs produced by $1x10^8$ bacteria. Cultures were acidified with phosphoric acid (H_3PO_4).

A


10.00
9.00
8.00
7.00
6.00
Viable count (log cfu/ml)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/5.0
7.6/4.5

B

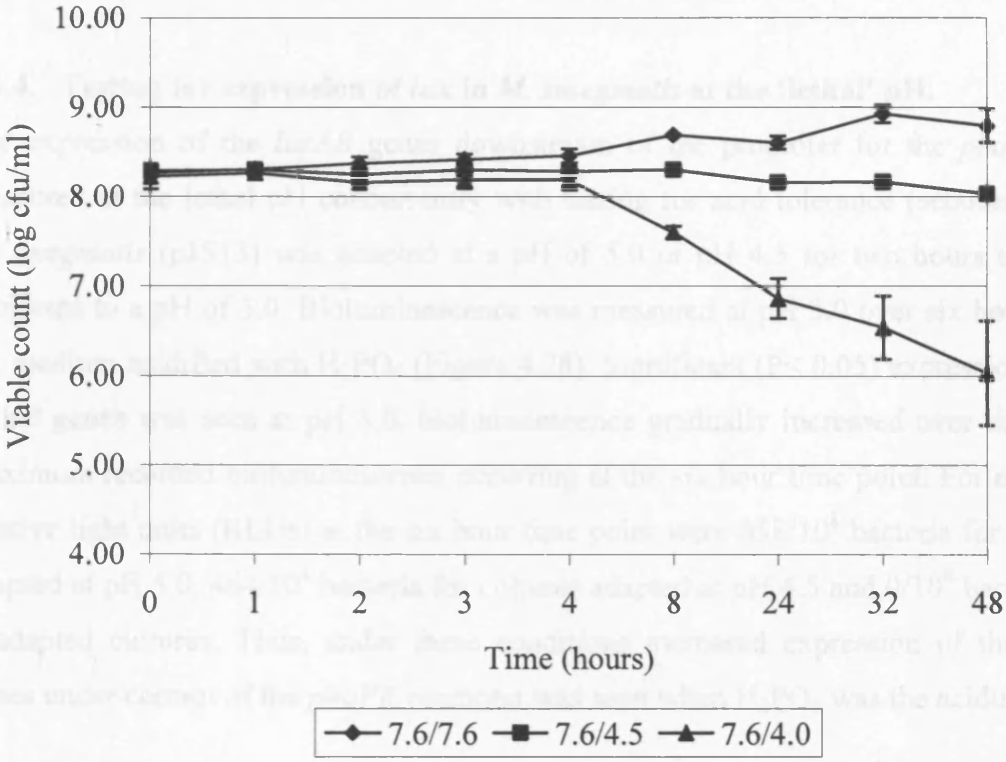

350
300
250
200
150
100
50
0
Light (RLU per 1x10^8 bacteria)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/5.0
7.6/4.5

(RLUs) at the twenty four hour time point were $238/10^8$ bacteria for cultures adapted at pH 5.0, $65/10^8$ bacteria for cultures adapted at pH 4.5 and $2/10^8$ bacteria for unadapted cultures. The levels of light produced appeared to be higher at pH 5.0 than at pH 4.5 throughout the most of the experiment (Figure 4.26). However, significant ($P< 0.05$) expression of *luxAB* was induced at twenty four hours to thirty two hours with only pH 4.5 adapted cultures. No light was detected after twenty four hours from the unadapted cultures. Exposing *M. smegmatis* (pJS13) at pH 5.0 or pH 4.5 with H_3PO_4 as the acidulant successfully resulted in the induction of *luxAB* expression downstream of the promoter for the *phoPR* gene, thus demonstrating that the promoter P*phoPR* is acid-inducible.

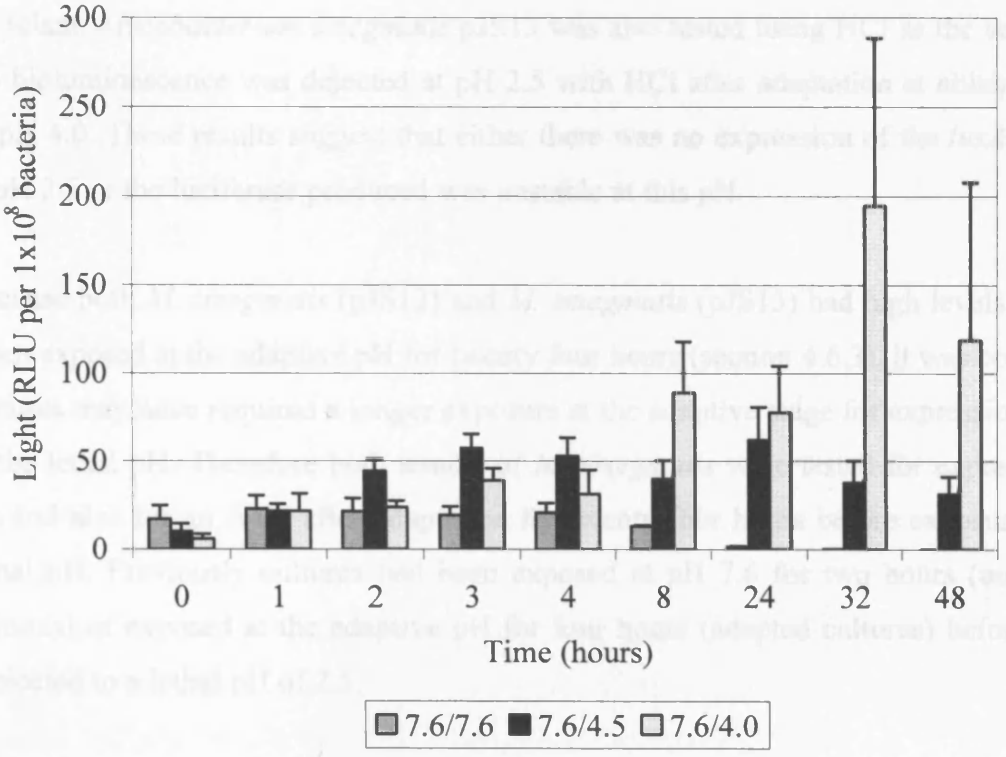
The experiments described above were repeated with *M. smegmatis* (pJS13) but using hydrochloric acid as the acidulant. Cultures were exposed to a pH of 4.5 or pH 4.0 or at a control pH of 7.6. Bioluminescence was measured and viable counts were done for the unadapted (pH 7.6) and adapted (pH 4.5 or pH 4.0) cultures over forty eight hours (Figure 4.27). Adapted cultures at pH 4.5 produced significantly ($P< 0.01$) higher levels of bioluminescence from two hours to twenty four hours. Cultures adapted at pH 4.0 also showed significant increases in bioluminescence at three hours to twenty four hours. The *lux* genes appeared to be expressed maximally at twenty four hours for cultures adapted at pH 4.5 and at thirty two hours for cultures adapted at pH 4.0 (Figure 4.27). Relative light units (RLUs) at thirty-two hours were $38/10^8$ bacteria for cultures adapted at pH 4.5, $195/10^8$ bacteria for cultures adapted at pH 4.0 and $0.22/10^8$ bacteria for unadapted cultures at pH 7.6. However, no significant ($P> 0.05$) increases in expression were seen at thirty two and forty eight hours for pH 4.5 or pH 4.0 adapted cultures when compared with the unadapted cultures. No light was produced from the unadapted cultures after twenty four hours. Thus, exposure of *M. smegmatis* (pJS13) to pH 4.5 or pH 4.0, using HCl as the acidulant, demonstrated that the promoter for the *phoPR* gene is acid-inducible.

Overall, both the promoters for the genes Rv2531c and *phoPR* were shown to be acid-inducible. Increased bioluminescence was seen using two types of acidulant, hydrochloric acid and phosphoric acid. Exposure of *M. smegmatis* (pJS12) at the adaptive pH resulted in maximum expression of the *luxAB* genes at the later time points,

A


10.00
9.00
8.00
7.00
6.00
5.00
4.00
Viable count (log cfu/ml)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/4.5
7.6/4.0

B


300
250
200
150
100
50
0
Light (RLU per 1x10^8 bacteria)
0
1
2
3
4
8
24
32
48
Time (hours)
7.6/7.6
7.6/4.5
7.6/4.0

that is twenty four to forty eight hours. Exposure of *M. smegmatis* (pJS13) at the adaptive pH resulted in expression of the *luxAB* genes during most of the experiment.

4.6.4. Testing for expression of *lux* in *M. smegmatis* at the 'lethal' pH.

The expression of the *luxAB* genes downstream of the promoter for the *phoPR* was measured at the lethal pH concurrently with testing for acid tolerance (section 4.6.2.). *M. smegmatis* (pJS13) was adapted at a pH of 5.0 or pH 4.5 for two hours and then subjected to a pH of 3.0. Bioluminescence was measured at pH 3.0 over six hours with the medium acidified with H_3PO_4 (Figure 4.28). Significant ($P< 0.05$) expression of the *luxAB* genes was seen at pH 3.0, bioluminescence gradually increased over time with maximum recorded bioluminescence occurring at the six hour time point. For example, relative light units (RLUs) at the six hour time point were $858/10^8$ bacteria for cultures adapted at pH 5.0, $464/10^8$ bacteria for cultures adapted at pH 4.5 and $0/10^8$ bacteria for unadapted cultures. Thus, under these conditions increased expression of the *luxAB* genes under control of the *phoPR* promoter was seen when H_3PO_4 was the acidulant.

Bioluminescence was also measured in *M. smegmatis* (pJS13) at pH 2.5 over 6 hours. Cultures were adapted at pH 5.0 or pH 4.5 for two hours and exposed to a pH 2.5 obtained with H_3PO_4. No light was detected over six hours at pH 2.5 with H_3PO_4 as the acidulant. *Mycobacterium smegmatis*:pJS13 was also tested using HCl as the acidulant. No bioluminescence was detected at pH 2.5 with HCl after adaptation at either pH 4.5 or pH 4.0. These results suggest that either there was no expression of the *luxAB* genes at pH 2.5 or the luciferase produced was unstable at this pH.

Because both *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) had high levels of light when exposed at the adaptive pH for twenty four hours (section 4.6.3), it was concluded cultures may have required a longer exposure at the adaptive stage for expression of *lux* at the lethal pH. Therefore both strains of *M. smegmatis* were tested for expression of *lux* and also for an ATR after adaptation for twenty four hours before exposure at the lethal pH. Previously cultures had been exposed at pH 7.6 for two hours (unadapted cultures) or exposed at the adaptive pH for four hours (adapted cultures) before being subjected to a lethal pH of 2.5.

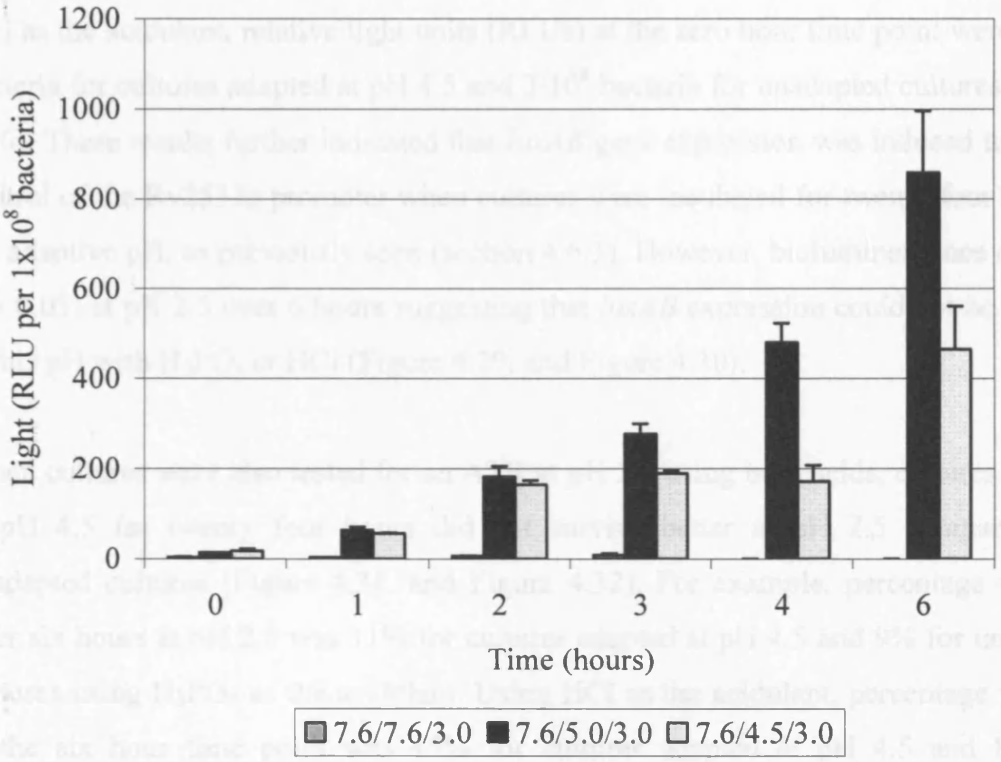


Figure 4.28. Testing *M. smegmatis* (pJS13) for expression of *lux* at pH 3.0 obtained with phosphoric acid.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 for two hours before exposure to a lethal pH of 3.0. The graph shows the amount of light produced by *M. smegmatis* (pJS13) over 6 hours. The values have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the relative light units produced by $1x10^8$ bacteria. Cultures were acidified with phosphoric acid (H_3PO_4).

Testing was done using phosphoric acid or hydrochloric acid as the acidulants. Bioluminescence and viability were measured over six hours at pH 2.5. When *M. smegmatis* (pJS12) was tested with H_3PO_4, high levels of light were detected at pH 2.5 at time zero from cultures adapted at pH 4.5 compared with unadapted cultures (Figure 4.29). For example, relative light units (RLUs) at the zero hour time point were $201/10^8$ bacteria for cultures adapted at pH 4.5 and $23/10^8$ bacteria for unadapted cultures. Using HCl as the acidulant, relative light units (RLUs) at the zero hour time point were $18/10^8$ bacteria for cultures adapted at pH 4.5 and $3/10^8$ bacteria for unadapted cultures (Figure 4.30). These results further indicated that *luxAB* gene expression was induced under the control of the Rv2531c promoter when cultures were incubated for twenty four hours at the adaptive pH, as previously seen (section 4.6.3). However, bioluminescence declined ($P > 0.05$) at pH 2.5 over 6 hours suggesting that *luxAB* expression could not be induced at this pH with H_3PO_4 or HCl (Figure 4.29. and Figure 4.30).

When cultures were also tested for an ATR at pH 2.5 using both acids, cultures adapted at pH 4.5 for twenty four hours did not survive better at pH 2.5 compared with unadapted cultures (Figure 4.31. and Figure 4.32). For example, percentage viability after six hours at pH 2.5 was 11% for cultures adapted at pH 4.5 and 9% for unadapted cultures using H_3PO_4 as the acidulant. Using HCl as the acidulant, percentage viability at the six hour time point was 15% for cultures adapted at pH 4.5 and 81% for unadapted cultures.

The results in the two paragraphs above demonstrate that adaptation for twenty four hours does not increase the expression of the *lux* system downstream of the Rv2531c promoter at pH 2.5 and also under these conditions, *M. smegmatis* (pJS12) does not possess an ATR at pH 2.5 with either H_3PO_4 or HCl.

Testing was repeated with *M. smegmatis* (pJS13) using only H_3PO_4 to acidify the medium. When cultures were adapted at pH 4.5 for twenty four hours prior to exposure at a lethal pH of 2.5, bioluminescence was detected at pH 2.5 over six hours. Bioluminescence appeared to increase from time zero at pH 2.5 and maximum recorded expression appeared to occur at four hours (Figure 4.33). For example, relative light units (RLUs) at the four hour time point were $211/10^8$ bacteria for cultures adapted at pH 4.5 and $0.21/10^8$ bacteria for unadapted cultures. However, significant ($P < 0.05$)

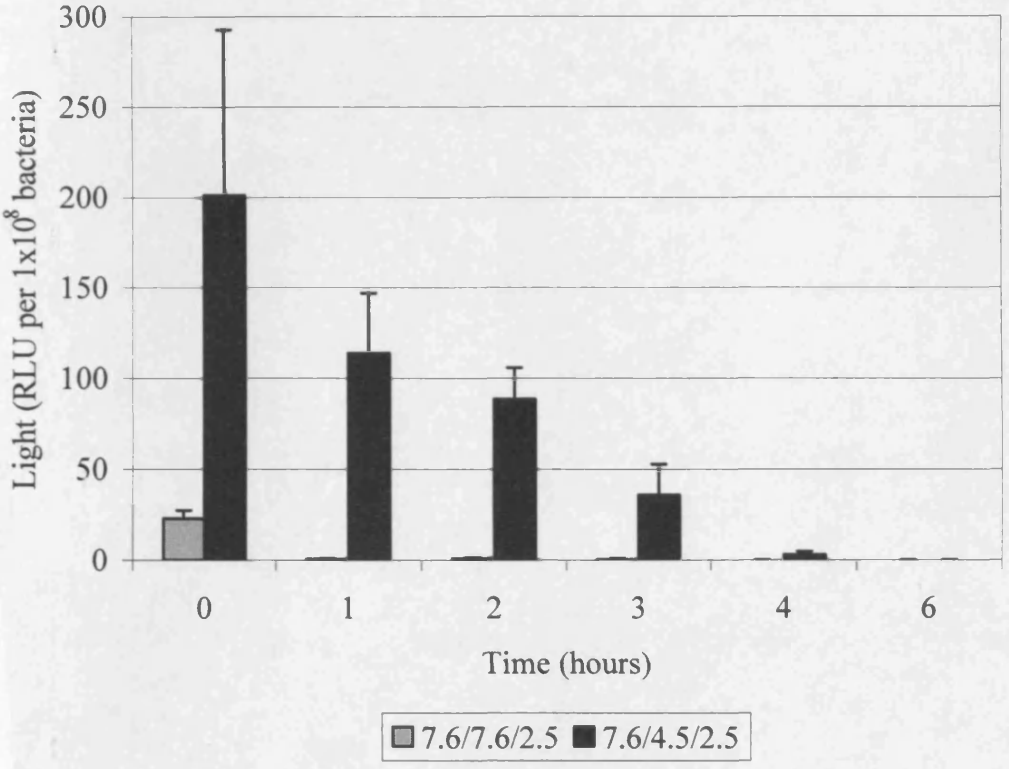


Figure 4.29. Testing *M. smegmatis* (pJS12) for expression of *lux* at the lethal pH obtained with phosphoric acid.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. Results show the amount of light produced at the lethal pH by *M. smegmatis* (pJS12) over 6 hours. The values have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the relative light units produced by $1x10^8$ bacteria. Cultures were acidified with phosphoric acid (H_3P0_4). All values represent the mean ± S.E.M. of four experiments.

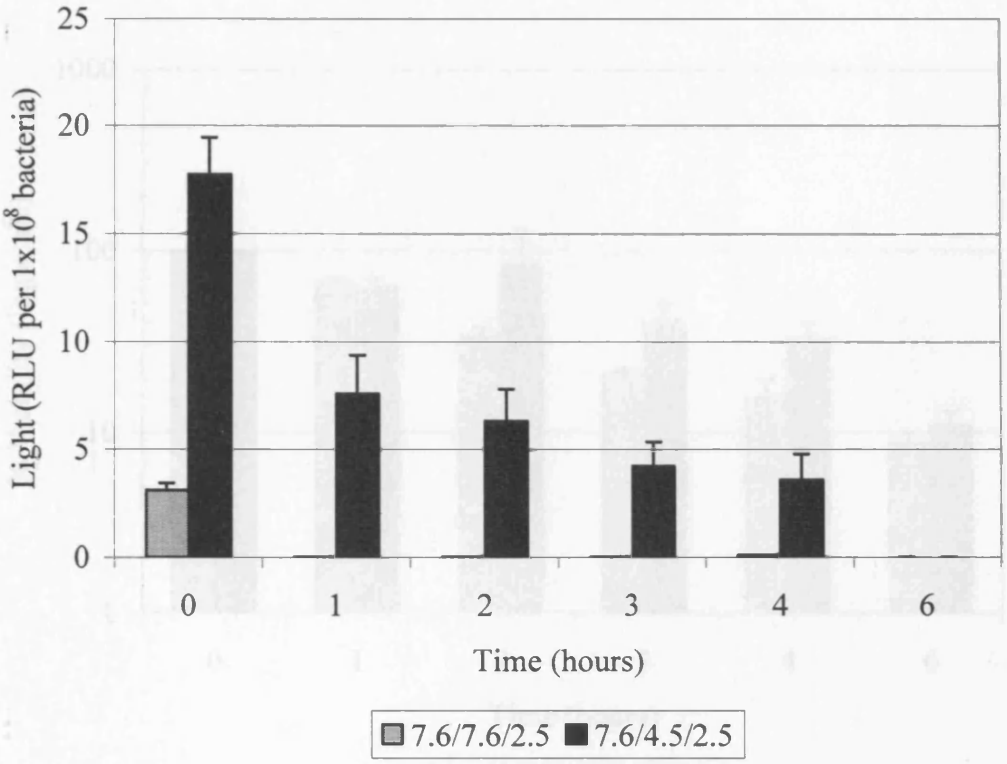


Figure 4.30. Testing *M. smegmatis* (pJS12) for expression of *lux* at the lethal pH obtained with hydrochloric acid.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. Results show the amount of light produced at the lethal pH by *M. smegmatis* (pJS12) over 6 hours. The values have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the relative light units produced by $1x10^8$ bacteria. Cultures were acidified with hydrochloric acid (HCl). All values represent the mean ± S.E.M. of four experiments.

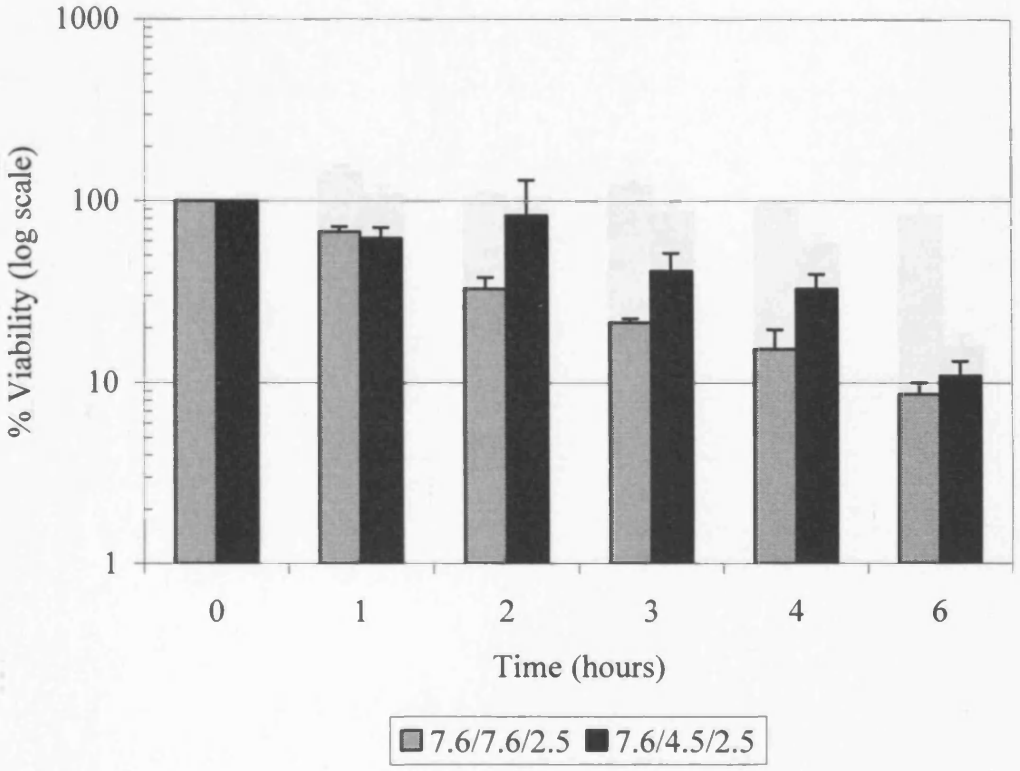


Figure 4.31. Testing *M. smegmatis* (pJS12) for an ATR after 24 hours with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS12) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3P0_4). Log cfu/ml mean values for cultures grown at pH 7.6 or 4.5 at 0 time are 8.36 and 7.69, respectively.

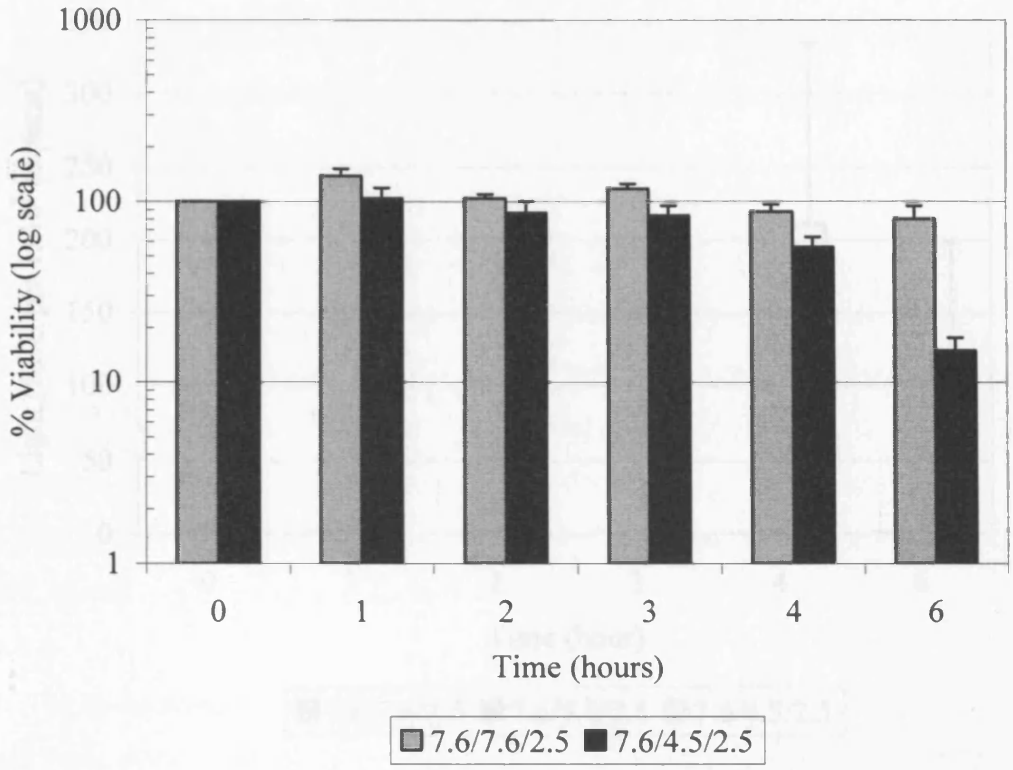


Figure 4.32. Testing *M. smegmatis* (pJS12) for an ATR after 24 hours with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS12) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6 or 4.5 at 0 time are 9.07 and 8.31, respectively.

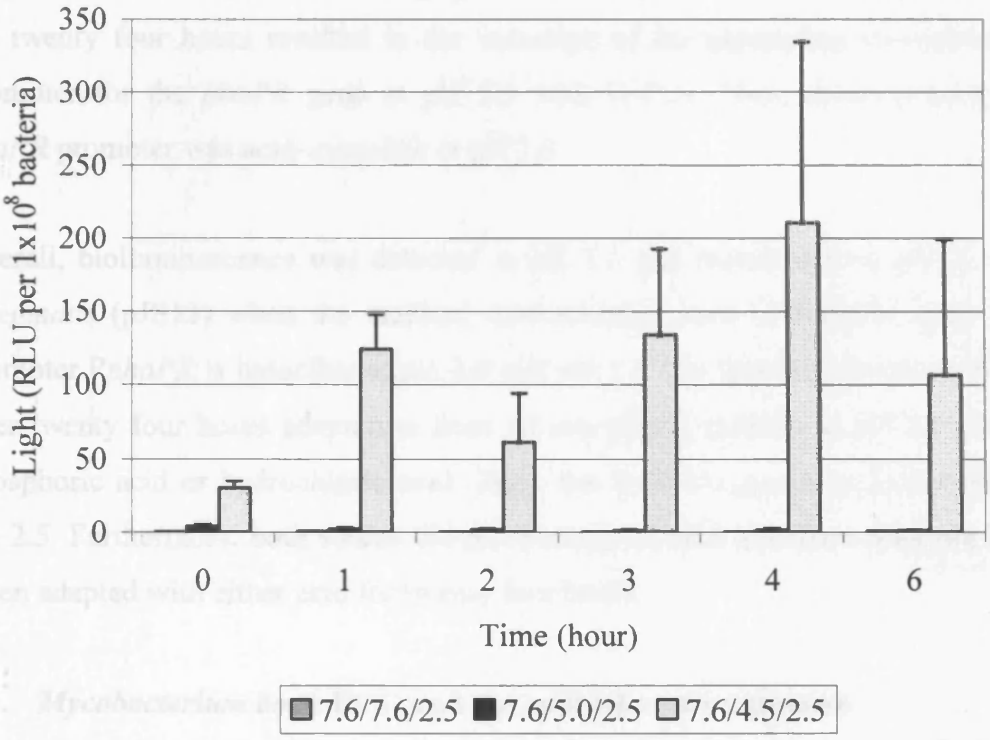


Figure 4.33. Testing *M. smegmatis* (pJS13) for expression of *lux* at the lethal pH obtained with phosphoric acid.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. The graph shows the amount of light produced at the lethal pH by *M. smegmatis* (pJS13) over 6 hours. The relative light units (RLUs) have been normalised to account for the differences in the viable counts of the cultures at each time point and results are represented as the RLUs produced by $1x10^8$ bacteria. Cultures were acidified with phosphoric acid (H_3PO_4).

expression of light was only detected at one hour. No light was detected at pH 2.5 from cultures that had been adapted at pH 5.0. Cultures also did not exhibit an ATR at pH 2.5 after adaptation with H_3PO_4. Cultures adapted at pH 5.0 or 4.5 for twenty four hours did not survive better at pH 2.5 compared with unadapted cultures (Figure 4.34). Thus, demonstrating that *M. smegmatis* (pJS13) does not possess an ATR at pH 2.5 when adapted for twenty four hours. Although an ATR was not observed, adaptation at pH 4.5 for twenty four hours resulted in the induction of *lux* expression downstream of the promoter for the *phoPR* gene at pH 2.5 with H_3PO_4. Thus, demonstrating that the *phoPR* promoter was acid-inducible at pH 2.5.

Overall, bioluminescence was detected at pH 3.0 and temporarily at pH 2.5 from *M. smegmatis* (pJS13) when the medium was acidified with phosphoric acid. Thus, the promoter P*phoPR* is inducible at pH 3.0 and pH 2.5. No bioluminescence was detected after twenty four hours adaptation from *M. smegmatis* (pJS12) at pH 2.5 using either phosphoric acid or hydrochloric acid. Thus, the Rv2531c promoter is not inducible at pH 2.5. Furthermore, both strains did not possess an acid tolerance response at pH 2.5 when adapted with either acid for twenty four hours.

4.7. *Mycobacterium bovis* BCG and the acid tolerance response.

Work was done with BCG to test for an acid tolerance response (ATR). The two plasmids pJS12 and pJS13 containing the *luxAB* genes downstream of the promoters for the Rv2531c and *phoPR* genes respectively were introduced into BCG. By observing the increased expression of the *lux* reporter downstream of the promoters of these two genes, it is possible to test as to whether Rv2531c and *phoPR* could be involved in acid tolerance. Two different acidulants were used; hydrochloric acid (HCl) and phosphoric acid (H_3PO_4).

4.7.1. Growth curve of *M. bovis* BCG

A growth curve was done to assess growth of BCG at pH 7.6 and to determine the length of time required for approximately $1x10^6$ bacteria to reach $1x10^8$ based on experiments done by Foster and Hall (1990). BCG was inoculated into 7H9 broth at pH 7.6 and 1 ml samples were taken after 0, 24, 48, 72, 96, 144, 168, 192, 216 and 240 hours. Viable counting and optical density readings at 550 nm were measured at each time point. Results suggested that BCG should be grown for approximately six

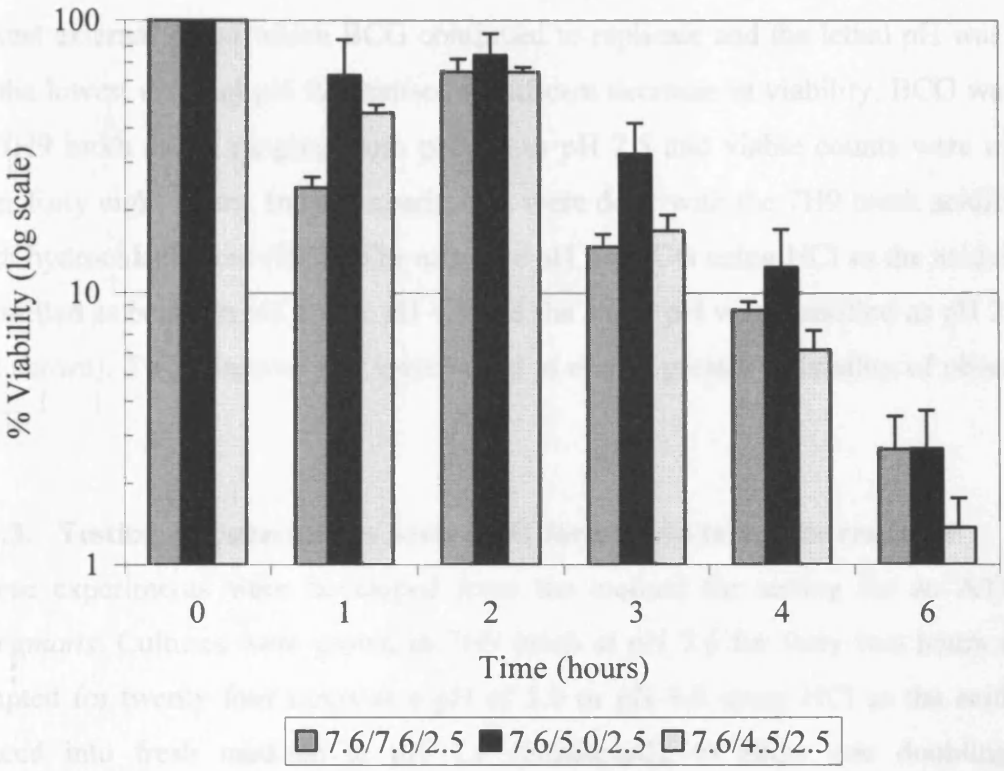


Figure 4.34. Testing *M. smegmatis* (pJS13) for an ATR after 24 hours with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 2.5. The graph shows the percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS13) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.0 or 4.5 at 0 time are 8.72, 8.07 and 7.86, respectively.

days at pH 7.6 to attain approximately $1x10^8$ bacteria (Figure 4.35). Consequently, BCG was grown for six days at pH 7.6 prior to testing BCG for an acid tolerance response.

4.7.2. Survival of *Mycobacterium bovis* BCG over a range of pH.

To identify the adaptive and lethal pHs to test for an acid tolerance response with BCG, the survival of BCG was tested over a range of pH. The adaptive pH was defined as the lowest external pH at which BCG continued to replicate and the lethal pH was defined as the lowest external pH that caused significant decrease in viability. BCG was grown in 7H9 broth at pH ranging from pH 7.6 to pH 2.5 and viable counts were measured over forty eight hours. Initial experiments were done with the 7H9 broth acidified only with hydrochloric acid (HCl). The adaptive pH for BCG using HCl as the acidulant was identified as between pH 5.0 or pH 4.0 and the lethal pH was identified as pH 2.5 (Data not shown). Two adaptive pHs were tested to ensure greater possibility of observing an ATR.

4.7.3. Testing *Mycobacterium bovis* BCG for an acid tolerance response.

These experiments were developed from the method for testing for an ATR in *M. smegmatis*. Cultures were grown in 7H9 broth at pH 7.6 for forty two hours and then adapted for twenty four hours at a pH of 5.0 or pH 4.0 using HCl as the acidulant or placed into fresh medium at pH 7.6 (unadapted), to allow one doubling before challenging cultures at a lethal pH of 2.5. Viable counts were done over a period of forty eight hours. However, these experiments failed to exhibit an ATR with BCG. Experiments were repeated on several occasions but cultures that had been adapted at a pH of 5.0 or pH 4.0 showed extensive clumping of the cells at pH 2.5 after incubation for twenty four hours. This characteristic was not demonstrated at pH 2.5 with unadapted cultures and these cells died normally. This suggested under these conditions a change had occurred to the membrane surface of the cell in BCG. Although the clumping indicated that an acid adaptive response was occurring, it prevented the quantitation of an acid tolerance response. To reduce the clumping, cultures were grown at pH 6.6-6.8, the standard pH of 7H9 broth before shifting to the adaptation pH and then exposing cultures at pH 2.5. However, adapted cultures continued to clump at pH 2.5, whilst unadapted cultures remained clump-free.

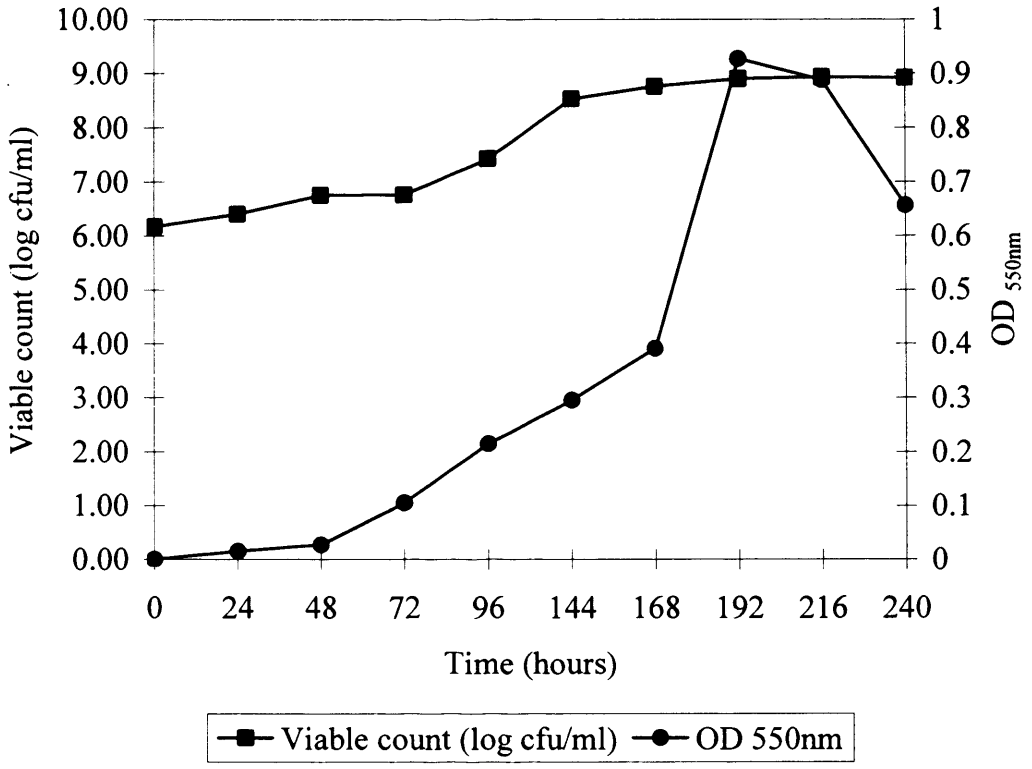


Figure 4.35. Growth curve of *Mycobacterium bovis* BCG.

A growth curve of BCG at pH 7.6 was done to assess growth. BCG was inoculated into 7H9 broth at pH 7.6 and 1 ml samples were taken at 0, 24, 48, 72, 96, 144, 168, 192, 216 and 240 hours. Viability and optical density at 550nm was measured at each time point.

Since no measurements could be taken using these extremely clumped bacteria, the method for testing for an ATR with BCG was further modified. One parameter was to raise the lethal pH to 3.0 to try and reduce the clumping. When this was done, no clumping was observed with cultures exposed at pH 3.0 following adaptation. However, the viable counts obtained at time zero at pH 3.0 were lower than expected, should have been approximately at 10^8 but were much less and therefore cultures were grown for a period of six days at pH 7.6 to attain higher counts of bacteria (section 4.7.1. and Figure 4.35). The experiments to test for the survival of BCG over a range of pH were repeated and new adaptive and lethal pHs were chosen using two acidulants, hydrochloric acid and phosphoric acid (section 4.7.2.). Previous adaptive pHs identified with BCG using HCl were not so clear cut. The adaptive pH for BCG using HCl as the acidulant was identified as between pH 6.6 and pH 5.0 and the lethal pH was identified as pH 3.0 (Table 4.4.a.). The adaptive pH for BCG using H_3PO_4 as the acidulant was identified between pH 4.5 and pH 4.0 and the lethal pH was identified as pH 3.0 (Table 4.4.b.). Although these pHs were chosen, they were however again not so clear cut as those for *M. smegmatis*. These pHs were used to test for an ATR with the recombinant BCG in the next set of experiments.

The new adaptive and lethal pHs identified from the survival of BCG over a range of pH (section 4.7.2) were used to test for an acid tolerance response in BCG (pJS12) and BCG (pJS13) using HCl or H_3PO_4. BCG (pJS12) was grown in 7H9 broth at the control pH of 7.6 and adapted at a pH of 6.6 and pH 5.0 using HCl and incubated for twenty four hours before cultures were exposed to a lethal pH of 3.0 using HCl as the acidulant. Cultures adapted at a pH of 6.6 survived significantly ($P< 0.01$) better at pH 3.0 compared with unadapted cultures at all time points (Figure 4.36). For example, percentage viability at the six hour time point was 85% for cultures adapted at pH 6.6 and 54% for unadapted cultures. However, cultures adapted at pH 5.0 did not survive significantly ($P> 0.05$) better at pH 3.0. These results demonstrate adaptation at pH 6.6 with BCG (pJS12) leads to protection at a lethal pH of 3.0 using HCl as the acidulant, thus an ATR is observed but exposure to pH 5.0 does not result in protection at pH 3.0; hence no ATR.

The above experiments were repeated with BCG (pJS12) using different adaptive pHs to see if an ATR could still be elicited (Figure 4.37). Since adaptation at

Experiment 1.

Time (hrs)	0	6	24	48
pH				
7.6	6.58	6.48	6.84	6.93
6.6	6.31	6.25	6.38	6.43
6	6.56	6.41	6.45	6.17
5	6.57	6.05	6.45	6.38
4.5	6.43	6.30	6.48	6.32
4	6.50	6.47	6.38	6.28
3.5	6.48	6.36	6.05	5.11
3	6.44	6.45	5.69	4.76
2.5	6.30	6.32	5.83	4.67

Experiment 2.

Time (hrs)	0	6	24	48
pH				
7.6	6.38	6.42	6.45	6.95
6.6	6.31	6.41	6.41	6.43
6	6.47	6.24	6.27	6.10
5	6.35	6.43	6.36	6.15
4.5	6.30	6.18	6.51	6.35
4	6.53	6.27	6.46	6.26
3.5	6.49	6.25	6.94	5.17
3	6.45	6.13	5.69	4.60
2.5	6.36	6.19	5.89	5.04

Table 4.4.a. Survival of *Mycobacterium bovis* BCG over a range of pH.

Results show the log colony forming units/ml for BCG over 48 hours after incubation at each pH. Cultures were acidified with hydrochloric acid (HCl). All values within each experiment are the mean of two counts taken from a single sample at the appropriate time point.

Experiment 1.

Time (hrs)	0	6	24	48
pH				
7.6	6.28	6.45	6.55	6.89
6.6	6.18	6.20	6.23	6.38
6	6.33	6.40	6.41	6.21
5	6.28	6.40	6.56	6.22
4.5	6.25	6.29	6.50	6.40
4	6.40	6.50	6.60	6.23
3.5	6.52	6.45	6.39	6.01
3	5.96	6.43	6.09	5.92
2.5	6.42	6.17	6.08	5.43

Experiment 2.

Time (hrs)	0	6	24	48
pH				
7.6	6.32	6.36	6.63	7.12
6.6	6.09	6.55	6.27	6.56
6	6.23	6.49	6.22	6.21
5	6.16	6.39	6.44	6.21
4.5	6.30	6.40	6.45	6.30
4	6.07	6.35	6.56	6.24
3.5	6.29	6.41	6.28	6.00
3	6.29	6.24	6.10	5.92
2.5	6.16	6.31	5.94	5.27

Table 4.4.b. Survival of *Mycobacterium bovis* BCG over a range of pH.
Results show the log colony forming units/ml for BCG over 48 hours after incubation at each pH. Cultures were acidified with phosphoric acid (H_3PO_4). All values within each experiment are the mean of two counts taken from a single sample at the appropriate time point.

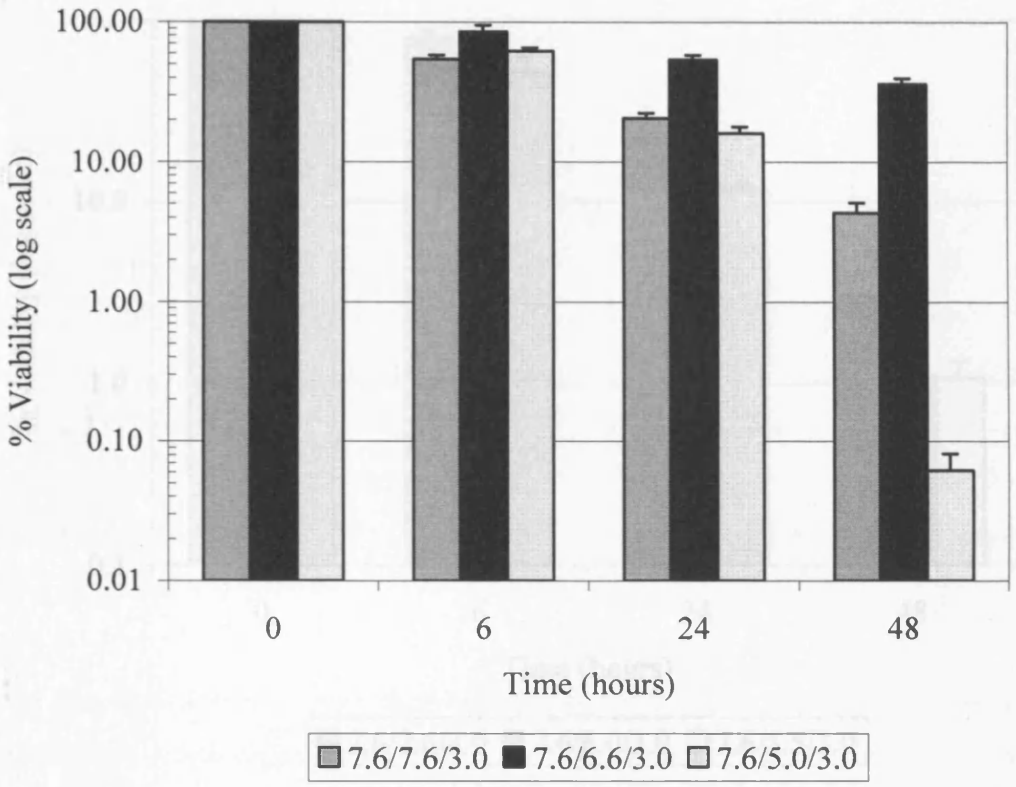


Figure 4.36. Testing BCG (pJS12) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 6.6 or pH 5.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS12) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 6.6 or 5.0 at 0 time are 7.80, 7.67 and 7.49, respectively.

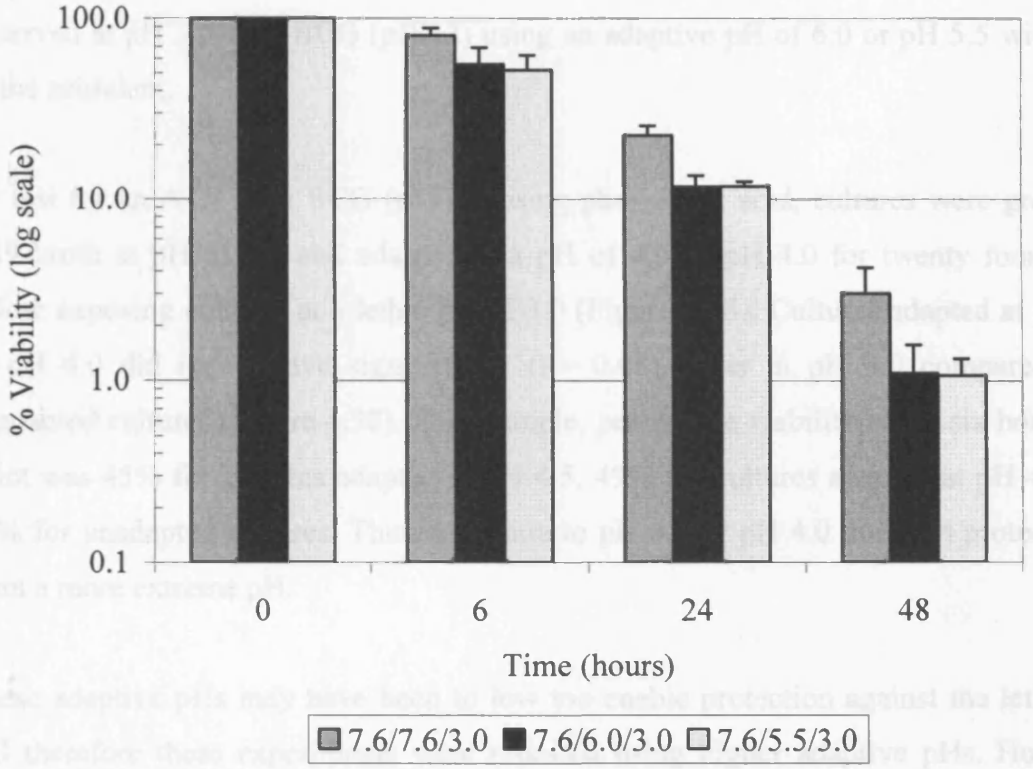


Figure 4.37. Testing BCG (pJS12) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 6.0 or pH 5.5 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS12) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 6.0 or 5.5 at 0 time are 7.64, 7.83 and 7.58 respectively.

pH 6.6 resulted in an ATR and protection at pH 3.0 but pH 5.0 did not, adaptation at pH 6.0 and pH 5.5 was tested. When cultures were adapted at these pHs, cultures did not survive significantly (P> 0.05) better at pH 3.0 compared with unadapted cultures over forty eight hours using HCl as the acidulant (Figure 4.37). For example, percentage viability at the six hour time point was 56% for cultures adapted at pH 6.0, 52% for cultures adapted at pH 5.5 and 81% for unadapted cultures. Thus, no acid tolerance is observed at pH 3.0 with BCG (pJS12) using an adaptive pH of 6.0 or pH 5.5 with HCl as the acidulant.

To test for an ATR with BCG (pJS12) using phosphoric acid, cultures were grown in 7H9 broth at pH of 7.6 and adapted at a pH of 4.5 or pH 4.0 for twenty four hours before exposing cultures at a lethal pH of 3.0 (Figure 4.38). Cultures adapted at pH 4.5 or pH 4.0 did not survive significantly (P> 0.05) better at pH 3.0 compared with unadapted cultures (Figure 4.38). For example, percentage viability at the six hour time point was 45% for cultures adapted at pH 4.5, 49% for cultures adapted at pH 4.0 and 27% for unadapted cultures. Thus, exposure to pH 4.5 or pH 4.0 does not protect cells from a more extreme pH.

These adaptive pHs may have been to low too enable protection against the lethal pH and therefore these experiments were repeated using higher adaptive pHs. However, when cultures were adapted at a higher pH of 5.5 or pH 5.0 for twenty four hours, cultures did not survive significantly (P> 0.05) better at a lethal pH of 3.0 compared with unadapted cultures when measured over forty eight hours (Figure 4.39). Percentage viability at the six hour time point was 45% for cultures adapted at pH 5.5, 42% for cultures adapted at pH 5.0 and 27% for unadapted cultures at pH 7.6 (Figure 4.39). These adapted cultures responded similarly at pH 3.0 to cultures adapted at a pH of 4.5 or pH 4.0 using H_3PO_4 (Figure 4.36). Thus, using H_3PO_4 as the acidulant, adaptation at various acidities between pH 4.0 and pH 5.5 did not provide protection against the lethal pH of 3.0 with BCG (pJS12).

BCG (pJS13) was tested for an ATR at pH 3.0 using HCl as the acidulant (Figure 4.40). BCG (pJS13) was grown in 7H9 broth at pH 7.6 for six days and adapted at pH 6.6 or pH 5.0 for twenty four hours before exposing cultures at a lethal pH of 3.0. Cultures adapted at a pH of 6.6 survived significantly (P< 0.05) better at pH

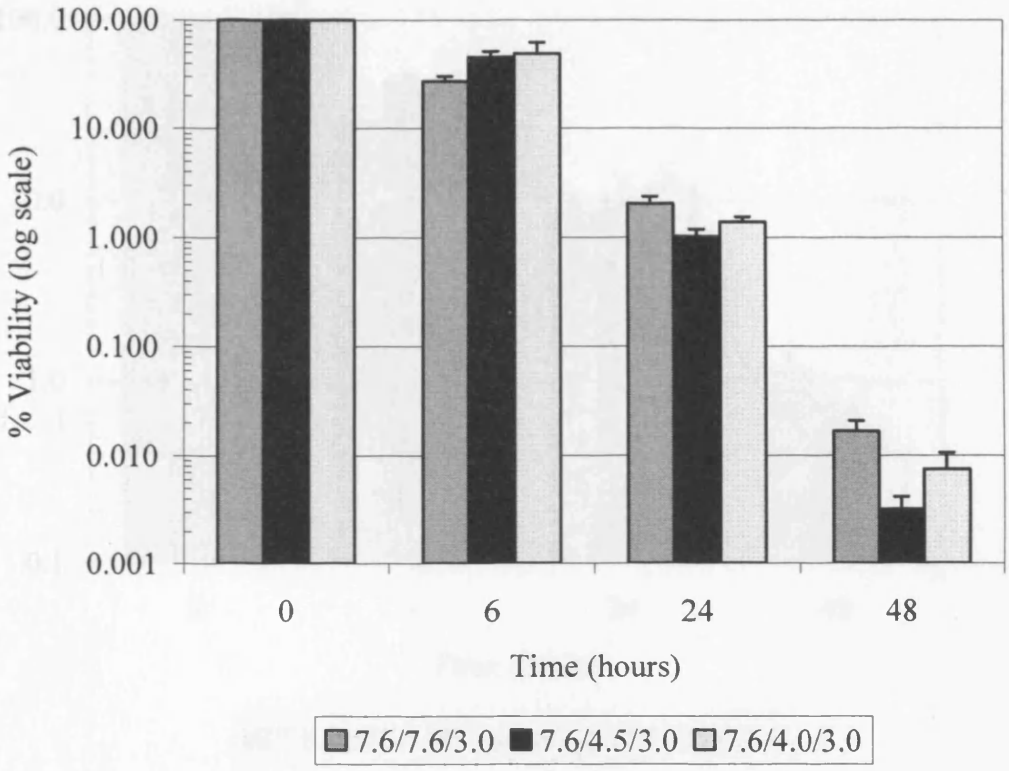


Figure 4.38. Testing BCG (pJS12) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or pH 4.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS12) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 4.5 or 4.0 at 0 time are 7.54, 7.35 and 7.08, respectively.

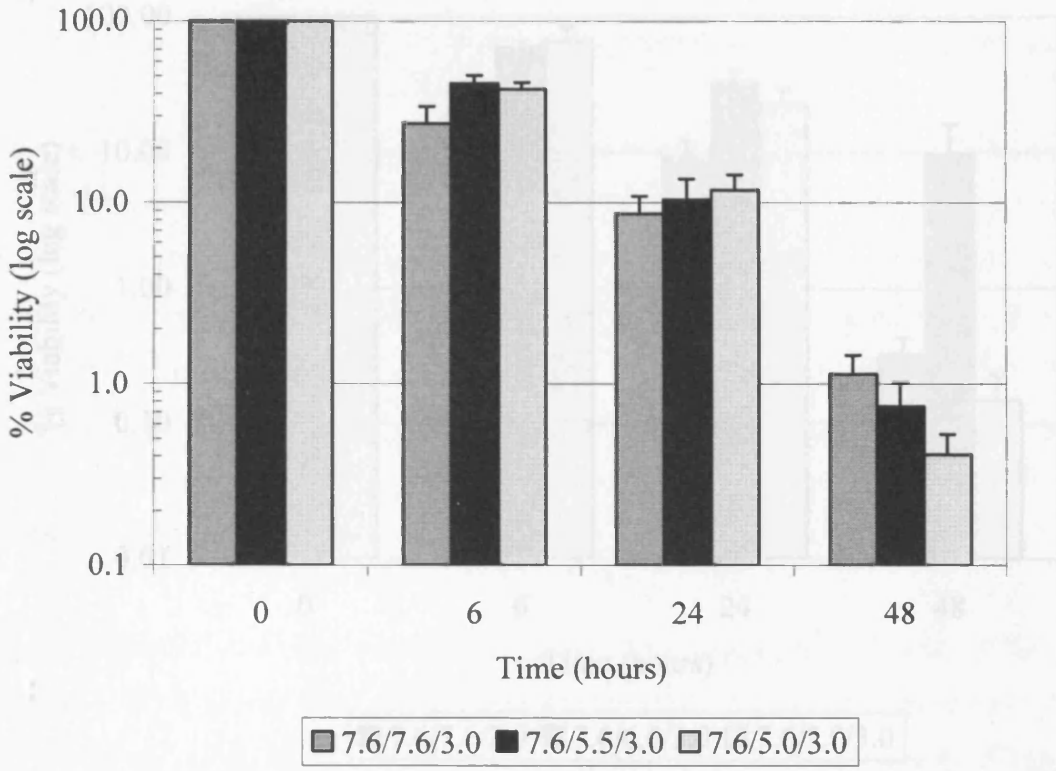


Figure 4.39. Testing BCG (pJS12) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.5 or pH 5.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS12) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.5 or 5.0 at 0 time are 7.99, 7.88, and 7.83, respectively.

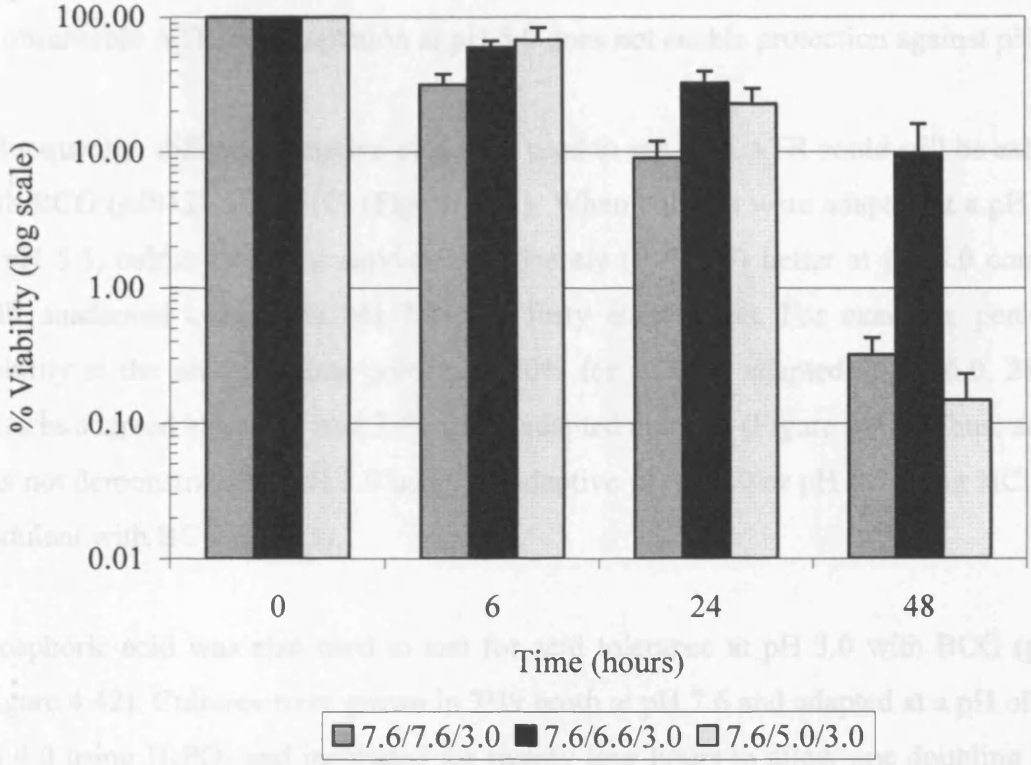


Figure 4.40. Testing BCG (pJS13) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 6.6 or pH 5.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS13) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 6.6 or 5.0 at 0 time are 7.97, 7.84 and 7.63, respectively.

3.0 at and after twenty four hours compared with unadapted cultures (Figure 4.40). For example, percentage viability at the twenty four hour time point was 32% for cultures adapted at pH 6.6 and 9% for unadapted cultures. Cultures adapted at pH 5.0 did not survive significantly ($P > 0.05$) better at pH 3.0 when compared to unadapted cultures (Figure 4.40). For example, percentage viability at six hours was 67% for cultures adapted at pH 5.0 and 31% for unadapted cultures. Thus, exposure at pH 6.6 resulted in an observable ATR but adaptation at pH 5.0 does not enable protection against pH 3.0.

Subsequently, different adaptive pHs were used to see if an ATR could still be exhibited with BCG (pJS13) using HCl (Figure 4.41). When cultures were adapted at a pH of 6.0 or pH 5.5, cultures did not survive significantly ($P > 0.05$) better at pH 3.0 compared with unadapted cultures at pH 7.6 over forty eight hours. For example, percentage viability at the six hour time point was 20% for cultures adapted at pH 6.0, 28% for cultures adapted at pH 5.5 and 23% for unadapted cultures (Figure 4.41). Thus, an ATR was not demonstrated at pH 3.0 using an adaptive pH of 6.0 or pH 5.5 using HCl as the acidulant with BCG (pJS13).

Phosphoric acid was also used to test for acid tolerance at pH 3.0 with BCG (pJS13) (Figure 4.42). Cultures were grown in 7H9 broth at pH 7.6 and adapted at a pH of 4.5 or pH 4.0 using H_3PO_4 and incubated for twenty four hours to allow one doubling before cultures were exposed at a lethal pH of 3.0. Samples of each culture were removed at 0, 6, 24 and 48 hours and viable counts were done. Cultures adapted at pH 4.5 or pH 4.0 did not survive significantly ($P > 0.05$) better at pH 3.0 compared with unadapted cultures at pH 7.6. For example, percentage viability at the six hour time point was 35% for cultures adapted at pH 4.5, 28% for cultures adapted at pH 4.0 and 28% for unadapted cultures (Figure 4.42). These results demonstrate that BCG (pJS13) does not possess an ATR at pH 3.0 following adaptation with H_3PO_4 as the acidulant. Thus, no protection is provided from adaptation.

The above experiments were repeated but using higher adaptive pHs of 5.5 or pH 5.0, in order to attain an ATR with H_3PO_4. Cultures adapted at a pH of 5.5 or pH 5.0 did not

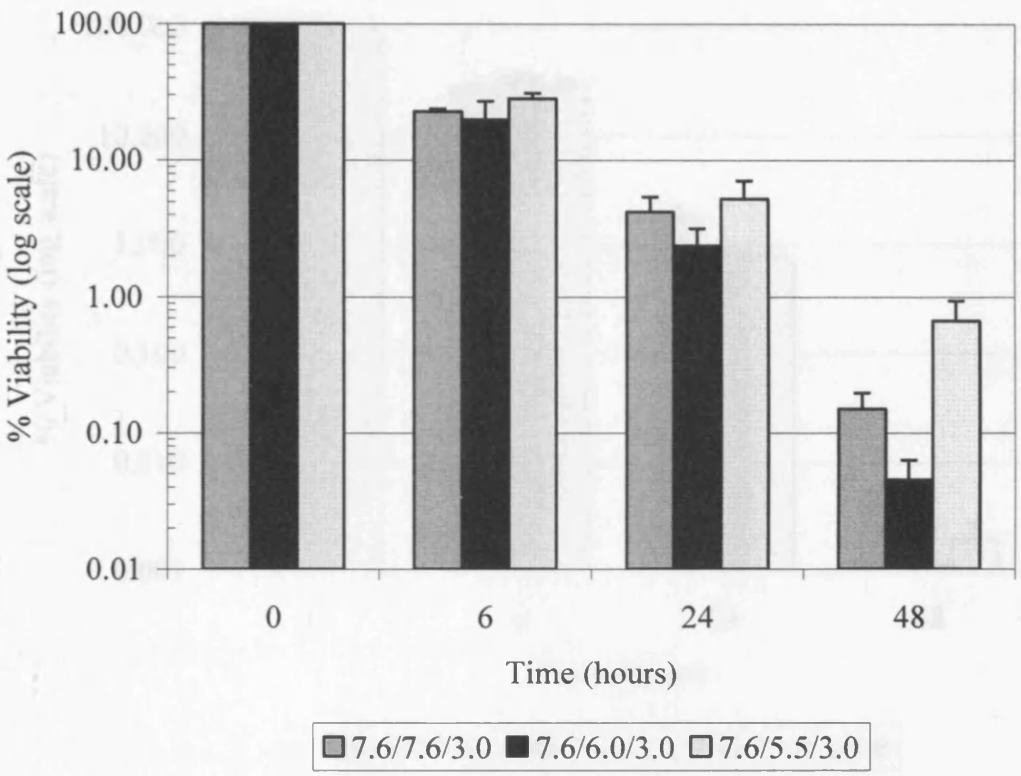


Figure 4.41. Testing BCG (pJS13) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 6.6 or pH 5.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS13) over 48 hours. All values represent the mean ± S.E.M. of four experiments Cultures were acidified with hydrochloric acid (HCl).Log cfu/ml mean values for cultures grown at pH 7.6, 6.6 or 5.0 at 0 time are 7.91, 8.18 and 7.66, respectively.

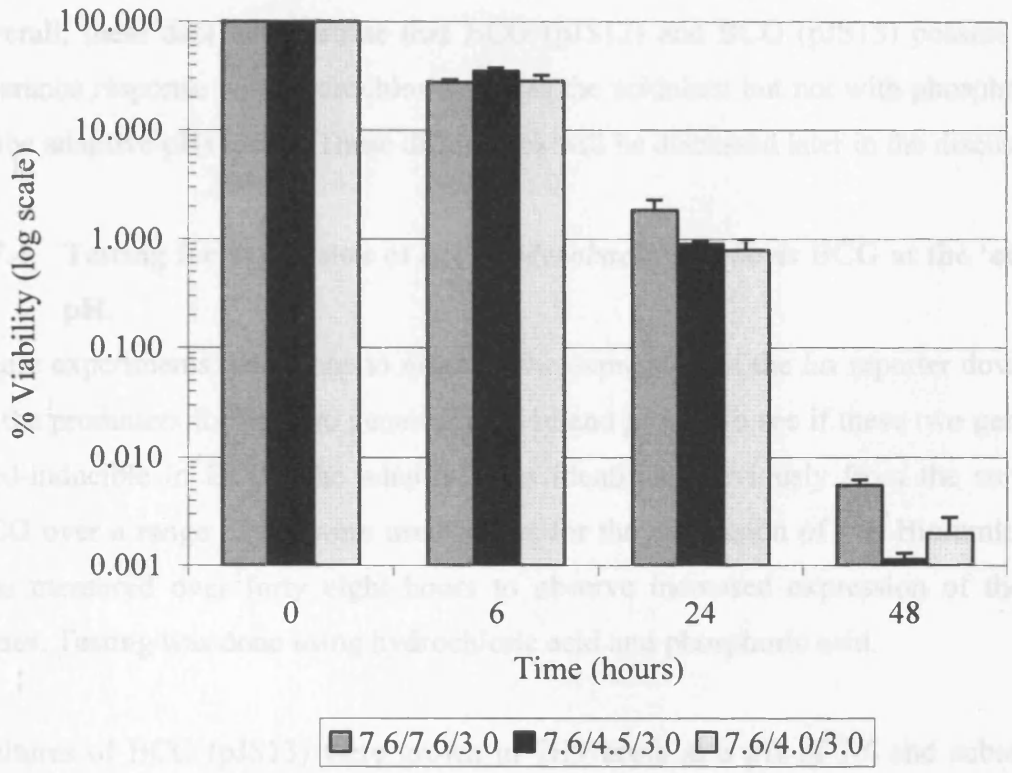


Figure 4.42. Testing BCG (pJS13) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or pH 4.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS13) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 4.5 or 4.0 at 0 time are 7.69, 7.49 and 7.43, respectively.

survive significantly (P> 0.05) better at pH 3.0 (Figure 4.43). For example, percentage viability at the six hour time point was 46% for cultures adapted at pH 5.5, 55% for cultures adapted at pH 5.0 and 18% for unadapted cultures at pH 7.6. These results demonstrate that adaptation at pH 5.5 or pH 5.0 does not result in acid tolerance when cells are further challenged at an extreme pH of 3.0 using H_3PO_4 as the acidulant.

Overall, these data demonstrate that BCG (pJS12) and BCG (pJS13) possess an acid tolerance response with hydrochloric acid as the acidulant but not with phosphoric acid at the adaptive pHs tested. These differences will be discussed later in the discussion.

4.7.4. Testing for expression of *lux* in *Mycobacterium bovis* BCG at the 'adaptive' pH.

These experiments were done to measure the expression of the *lux* reporter downstream of the promoters for the two genes, Rv2531c and *phoPR* to see if these two genes were acid-inducible in BCG. The adaptive pHs identified previously from the survival of BCG over a range of pH were used to test for the expression of *lux*. Bioluminescence was measured over forty eight hours to observe increased expression of the *luxAB* genes. Testing was done using hydrochloric acid and phosphoric acid.

Cultures of BCG (pJS13) were grown in 7H9 broth at a pH of 7.6 and subsequently exposed at a pH of 4.5 or pH 4.0 with H_3PO_4 or placed into fresh medium at the control pH of 7.6. Samples of each culture were removed at 0, 6, 24, and 48 hours and bioluminescence was measured immediately. No expression of the *lux* reporter occurred over forty eight hours, that is no light was detected from cultures adapted at pH 4.5 or pH 4.0 or from unadapted cultures using H_3PO_4 as the acidulant.

Experiments were repeated with BCG (pJS13) using HCl as the acidulant. Cultures were adapted at a pH of 6.6 or pH 5.0 but again no bioluminescence was detected in any of the cultures at any time point. Unfortunately, no time was available for further experiments with BCG (pJS12) to test for increased expression of *lux* at the adaptive stage, to see if the Rv2531c promoter upstream of *luxAB* was acid-inducible in BCG.

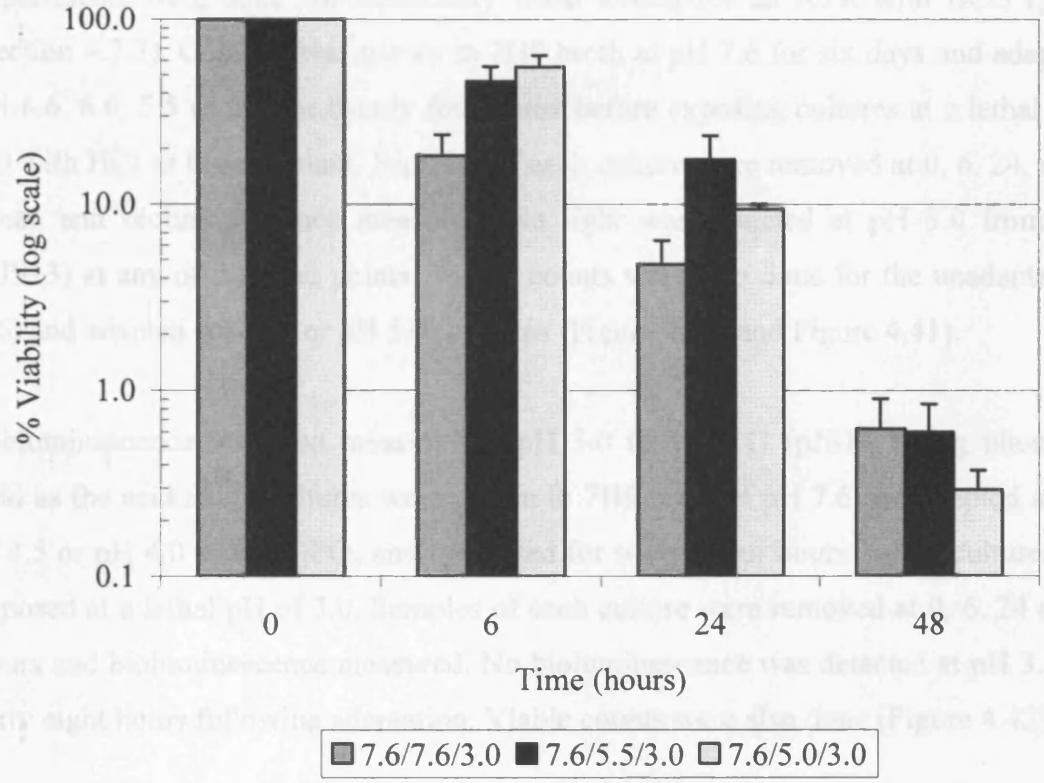


Figure 4.43. Testing BCG (pJS13) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.5 or pH 5.0 or grown at pH 7.6 (unadapted) for 24 hours before exposure to a lethal pH of 3.0. The graph shows the percentage viability (log scale) at the lethal pH for BCG (pJS13) over 48 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.5 or 5.0 at 0 time are 8.25, 7.97 and 7.79, respectively.

4.7.5. Testing for expression of *lux* in *Mycobacterium bovis* BCG at the 'lethal' pH.

The expression of the *luxAB* genes downstream of the promoter for the gene *phoPR* was also measured to see if the promoter was inducible at the lethal pH of 3.0. These experiments were done simultaneously when testing for an ATR with BCG (pJS13) (section 4.7.3). Cultures was grown in 7H9 broth at pH 7.6 for six days and adapted at pH 6.6, 6.0, 5.5 or 5.0 for twenty four hours before exposing cultures at a lethal pH of 3.0 with HCl as the acidulant. Samples of each culture were removed at 0, 6, 24, and 48 hours and bioluminescence measured. No light was detected at pH 3.0 from BCG (pJS13) at any of the time points. Viable counts were also done for the unadapted (pH 7.6) and adapted (pH 6.6 or pH 5.0) cultures (Figure 4.40. and Figure 4.41).

Bioluminescence was also measured at pH 3.0 from BCG (pJS13) using phosphoric acid as the acidulant. Cultures were grown in 7H9 broth at pH 7.6 and adapted at a pH of 4.5 or pH 4.0 using H_3PO_4 and incubated for twenty four hours before cultures were exposed at a lethal pH of 3.0. Samples of each culture were removed at 0, 6, 24 and 48 hours and bioluminescence measured. No bioluminescence was detected at pH 3.0 over forty eight hours following adaptation. Viable counts were also done (Figure 4.42).

The above experiments were repeated but using higher adaptive pHs of 5.5 or pH 5.0, to see if bioluminescence could be detected. No light was detected at pH 3.0 from cultures previously adapted at a pH of 5.5 or pH 5.0 or from unadapted cultures. Viable counts were also done (Figure 4.43). These results suggest that the promoter for the *phoPR* gene was not acid-inducible at pH 3.0 with H_3PO_4 as the acidulant.

Overall, no bioluminescence was detected from BCG (pJS13) when exposed at the lethal stage following adaptation with either phosphoric acid or hydrochloric acid.

Table 4.5 shows the overall summary of results for testing for an acid tolerance response in *M. smegmatis* (pJS12), *M. smegmatis* (pJS13), BCG (pJS12) and BCG (pJS13).

Table 4.5. Summary Of Acid Tolerance Response and Expression Results:

Strain	Acid	pH	5		4.5		4	
			3	2.5	3	2.5	3	2.5
M. smegmatis (pJS12)	H_3PO_4		x	o	x	o	-	-
M. smegmatis (pJS13)	H_3PO_4		o	o	o	o	-	-
M. smegmatis (pJS12)	HCl		-	-	-	o	-	o
M. smegmatis (pJS13)	HCl		-	-	-	o	-	o

Strain	Acid	pH	6.6	6.0	5.5	5.0	4.5	4.0
			3	3	3	3	3	3
BCG (pJS12)	H_3PO_4		-	-	x	x	x	x
BCG (pJS13)	H_3PO_4		-	-	x	x	x	x
BCG (pJS12)	HCl		o	x	x	x	-	-
BCG (pJS13)	HCl		o	x	x	x	-	-

o = represents ATR

x = represents No ATR

- = represents No data

Light grey shading represents *lux* expression at the adaptive pH.

Dark grey shading represents *lux* expression at the lethal pH.

A line across the box represents no *lux* expression at the lethal pH.

No ATR was seen when cells were adapted for 24 hours at the adaptive pH and challenged at the lethal pH.

CHAPTER 5: RESULTS.

5.1. Aim

The aim of the work described in this chapter was to construct a knockout mutation of the Rv2531c gene (putative decarboxylase) in *Mycobacterium smegmatis*, in order to see if this gene was involved in the acid tolerance response (ATR). This was done using a non-replicating suicide vector that also encoded the *sacRB* gene, which mediates sucrose sensitivity. Kanamycin and hygromycin resistance genes were also used as selectable markers for determining a double recombination event in *M. smegmatis*.

5.2. *Mycobacterium tuberculosis* Rv2531c Gene.

5.2.1. Design of the Rv2531c PCR primers from the *Mycobacterium tuberculosis* gene sequence.

To amplify the complete 2844 bp Rv2531c sequence from the *M. tuberculosis* cosmid MTCY159, PCR primers were designed from the 5' region of Rv2531c, identified from the *Mycobacterium tuberculosis* H37Rv genome project at The Sanger Centre (http://www.sanger.ac.uk) (Figure 5.1). Initially the forward primer was designed from the Rv2531c sequence starting from 1 bp and the reverse primer from 2844 bp. The forward primer Rv2531C-FOR and the reverse primer Rv2531C-REV were designed to amplify the 2844 bp Rv2531c gene from cosmid MTCY159. The complete 2844 bp Rv2531c fragment could not amplified. However, a fragment of approximately 1000 bp was continually amplified due to mispriming of the forward primer. Although the PCR of MTCY159 DNA was done using a number of different reaction parameters, including varying the concentration of DNA, annealing temperature, magnesium and primer concentration, this did not result in the amplification of the 2844 bp Rv2531c fragment. Thus, new primers were designed with restriction endonuclease sequence incorporated into the primers such that the amplified PCR product could be directly ligated into the multiple cloning site of the vector, pPCRScript. The forward primer Rv2531C-FOR1 and the reverse primer Rv2531C-REV2 were designed to amplify 2698 bp of the Rv2531c gene from cosmid MTCY159. A *Hind* III restriction endonuclease site was incorporated into the forward and the reverse primer. The restriction sites of the Rv2531c sequence from cosmid MTCY159 were identified using the 'MAP' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

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 61 ---------+---------+---------+---------+---------+---------+ 120
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241 ---------+---------+---------+---------+---------+---------+ 300
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301 ---------+---------+---------+---------+---------+---------+ 360
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481 ---------+---------+---------+---------+---------+---------+ 540
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781 ---------+---------+---------+---------+---------+---------+ 840
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1201 ---------+---------+---------+---------+---------+---------+ 1260
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1381 ---------+---------+---------+---------+---------+---------+ 1440
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1501 ---------+---------+---------+---------+---------+---------+ 1560
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     GACGCCACGTCGAGGAGATCACGCAGGATCTGCCGCATCTACCAGATTTCAGCGAGTTCG
2341 ---------+---------+---------+---------+---------+---------+ 2400
     CTGCGGTGCAGCTCCTCTAGTGCGTCCTAGACGGCGTAGATGGTCTAAAGTCGCTCAAGC

     ACCTTGCCTTCCGCCCCGACGACGCCAGCTCTTTCGGTGACATGCGGTCGGCTTTCTACG
2401 ---------+---------+---------+---------+---------+---------+ 2460
     TGGAACGGAAGGCGGGGCTGCTGCGGTCGAGAAAGCCACTGTACGCCAGCCGAAAGATGC

     CCGGCTACGAAGAGGCCGACCGTGAGTACGTGCAGATCGGCTTGGCCGGGCGCCGGCTGG
2461 ---------+---------+---------+---------+---------+---------+ 2520
     GGCCGATGCTTCTCCGGCTGGCACTCATGCACGTCTAGCCGAACCGGCCCGCGGCCGACC

     CTGAGGGCAAGACTCTGGTATCCACCACGTTCGTGGTGCCCTACCCGCCCGGCTTCCCGG
2521 ---------+---------+---------+---------+---------+---------+ 2580
     GACTCCCGTTCTGAGACCATAGGTGGTGCAAGCACCACGGGATGGGCGGGCCGAAGGGCC

     TACTGGTGCCGGGTCAACTGGTTTCCAAGGAGATCATCTACTTTCTTGCCCAGCTCGACG
2581 ---------+---------+---------+---------+---------+---------+ 2640
     ATGACCACGGCCCAGTTGACCAAAGGTTCCTCTAGTAGATGAAAGAACGGGTCGAGCTGC

     TCAAAGAGATCCACGGATACAACCCCGACCTGGGGTTGTCGGTGTTCACCCAGGCGGCAT
2641 ---------+---------+---------+---------+---------+---------+ 2700
     AGTTTCTCTAGGTGCCTATGTTGGGGCTGGACCCCAACAGCCACAAGTGGGTCCGCCGTA
```

```
     TGGCCCGGATGGAGGCCGCGCGCAACGCCGTCGCCACGGTAGGTGCCGCGCTGCCGGCCT
2701 ---------+---------+---------+---------+---------+---------+ 2760
     ACCGGGCCTACCTCCGGCGCGCGTTGCGGCAGCGGTGCCATCCACGGCGCGACGGCCGGA

     TCGAGGTACCGCGGGACGCTTCGGCTTTGAATGGCACCGTCAACGGCGACAGTGTGCTGC
2761 ---------+---------+---------+---------+---------+---------+ 2820
     AGCTCCATGGCGCCCTGCGAAGCCGAAACTTACCGTGGCAGTTGCCGCTGTCACACGACG

     AGGGGGTCGCCGAAGACGCGTGAC
2821 ---------+---------+---- 2844
     TCCCCCAGCGGCTTCTGCGCACTG
```

Figure 5.1. Nucleic acid sequence of the *M. tuberculosis* Rv2531c gene.

The forward and reverse primers used are shown in bold and underlined on the 2844 bp Rv2531c gene (putative decarboxylase) sequence from *M. tuberculosis* cosmid Y159 (MTCY159).

5.2.2. PCR to amplify the Rv2531c gene sequence.

Amplification of the Rv2531c gene sequence was done using primers Rv2531C-FOR1 and Rv2531C-REV2 using the polymerase chain reaction (Saiki *et al.*, 1988), as described in the materials and methods (section 2.5. and Table 2.3). The Rv2531c PCR product was visualised at approximately 2700 bp in a 0.7% (w/v) agarose gel (Figure 5.2). The band was excised from the agarose gel and the DNA was isolated using the Sephaglas Bandprep Kit.

5.2.3. Sequence of Rv2531c PCR product by automated sequencing.

The 1000 bp (see section 5.2.1.) and 2698 bp Rv2531c PCR products were sequenced using the ABI PRISM Ready Reaction Dye Terminator Cycle Sequencing Kit using primers Rv2531C-FOR/Rv2531C-REV and Rv2531C-FOR1 respectively. Sequence was analysed using AutoAssembler 1.4.0, Model 377 and the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991). Analysis of both sequences confirmed that the Rv2531c PCR product had been amplified in the region required (Figure 5.3. and Figure 5.4). The Rv2531C-FOR/Rv2531C-REV primers only amplified the last 1000 bp of the Rv2531c gene, due to mispriming of the forward primer. Therefore, the new forward primer Rv2531C-FOR1 was used to sequence the 2698 bp Rv2531c amplified fragment.

5.3. Construction of a *Mycobacterium smegmatis* Rv2531c gene knockout.

5.3.1. Cloning the Rv2531c PCR product into pPCRScript to produce pJS20.

The Rv2531c PCR product was cloned into pPCRScript (Figure 5.5). The 2698 bp Rv2531c amplified PCR product was digested with *Hind* III and ligated into the vector pPCRScript, which had also been digested with *Hind* III and dephosphorylated to prevent recirculisation of the vector DNA. The ligation product was introduced into *Escherichia coli* strain DH5α and transformants were selected on ampicillin. Unfortunately, no clones were identified and therefore, the blunt-ended Rv2531c amplified product was ligated directly into the *Srf* I site of pPCRScript using the PCR-Script Amp SK (+) cloning kit. The ligation product was transformed into Epicurian Coli XL1-Blue MRF' Kan supercompetent cells. Transformants were selected on 100

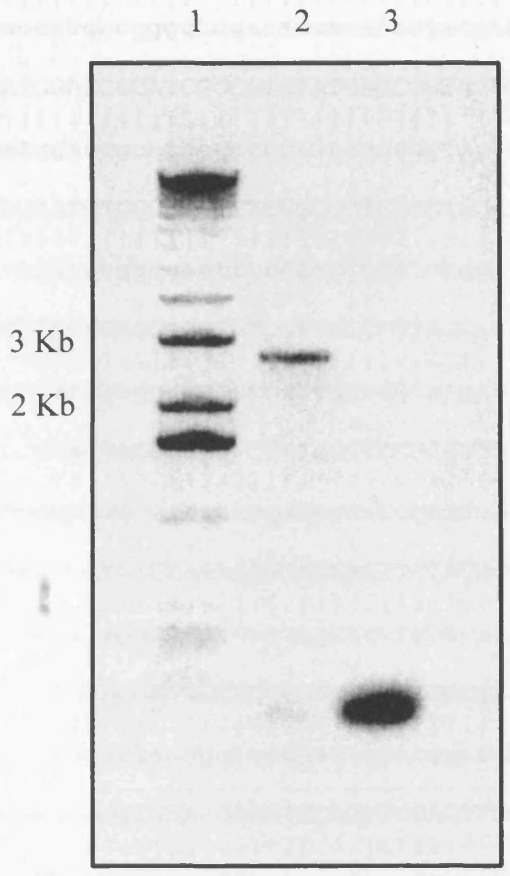


Figure 5.2. PCR to amplify the Rv2531c gene sequence.

Amplification of the Rv2531c gene sequence was done using primers Rv2531C-FOR1 and Rv2531C-REV2. Lane 1 contains DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb). Lane 2 contains Rv2531c PCR product at approximately 2700 bp. Lane 3 contains the negative control, the black band is primer dimer.

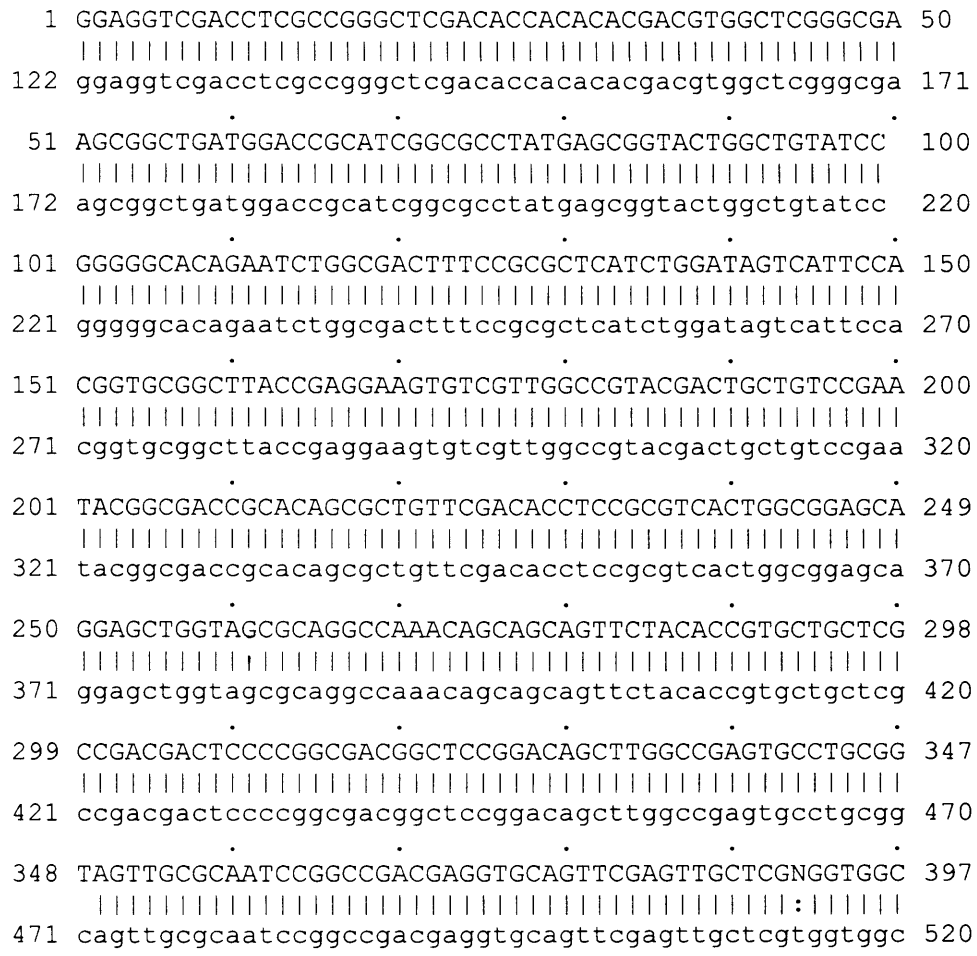


Figure 5.3. Forward sequence of the Rv2531c PCR product.

Nucleic acid sequence of the Rv2531c gene is shown. The forward sequence of the 2844 bp Rv2531c PCR amplified DNA fragment (top strand) is shown aligned against the Rv2531c gene of *M. tuberculosis* (bottom strand). The Rv2531c gene was sequenced using the forward primer Rv2531C-FOR1. This sequence has been matched to the original sequence (Figure 1.1) using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

```
 130 AAGCCTGGCGGTCCGATCAATTCGTGCTCGATCCGACGCGGCTCACCCTG 179
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2065 aagcctggcggtccgatcaattcgtgctcgatccgacgcggctcaccctg 2114
           .         .         .         .         .
 180 TTTATCGGGGCGACCGGGATGAACGGGTACGACTTCCGCGAGAAGATCCT 229
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2115 tttatcggggcgaccgggatgaacgggtacgacttccgcgagaagatcct 2164
           .         .         .         .         .
 230 GATGGAGCGATTCGGCATCCAGATCAACAAAACGTCTATCAACAGCGTGT 279
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2165 gatggagcgattcggcatccagatcaacaaaacgtctatcaacagcgtgt 2214
           .         .         .         .         .
 280 TGCTGATCTTCACGATCGGCGTCACCTGGTCGAGCGTGCACTATCTGCTC 329
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2215 tgctgatcttcacgatcggcgtcacctggtcgagcgtgcactatctgctc 2264
           .         .         .         .         .
 330 GATGTGTTGCGTCGGGTGGCGATCGATCTGGACCGCAGCCAGAAGGCGGC 379
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2265 gatgtgttgcgtcgggtggcgatcgatctggaccgcagccagaaggcggc 2314
           .         .         .         .         .
 380 CAGCGGGGCCGACCTTGCTCTACACCGACGCCACGTCGAGGAGATCACGC 429
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2315 cagcggggccgaccttgctctacaccgacgccacgtcgaggagatcacgc 2364
           .         .         .         .         .
 430 AGGATCTGCCGCATCTACCAGATTTCAGCGAGTTCGACCTTGCCTTCCGC 479
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2365 aggatctgccgcatctaccagatttcagcgagttcgaccttgccttccgc 2414
           .         .         .         .         .
 480 CCCGACGACGCCAGCTCTTTCGGTGACATGCGGTCGGCTTTCTACGCCGG 529
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2415 cccgacgacgccagctctttcggtgacatgcggtcggctttctacgccgg 2464
           .         .         .         .         .
 530 CTACGAAGAGGCCGACCGTGAGTACGTGCAGATCGGCTTGGCCGGGCGCC 579
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2465 ctacgaagaggccgaccgtgagtacgtgcagatcggcttggccgggcgcc 2514
           .         .         .         .         .
 580 GGCTGGCTGAGGGCAAGACTCTGGTATCCACCACGTTCGTGGTGCCCTAC 629
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2515 ggctggctgagggcaagactctggtatccaccacgttcgtggtgccctac 2564
           .         .         .         .         .
 630 CCGCCCGGCTTCCCGGTACTGGTGCCGGGTCAACTGGTTTCCAAGGAGAT 679
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2565 ccgcccggcttcccggtactggtgccgggtcaactggtttccaaggagat 2614
           .         .         .         .         .
 680 CATCTACTTTCTTGCCCAGCTCGACGTCAAAGAGATCCACGGATACAACC 729
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2615 catctactttcttgcccagctcgacgtcaaagagatccacggatacaacc 2664
           .         .         .         .         .
 730 CCGACCTGGGGTTGTCGGTGTTCACCCAGGCGGCATTGGCCCGGATGGAG 779
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
2665 ccgacctggggttgtcggtgttcacccaggcggcattggcccggatggag 2714
```

Figure 5.4. Reverse sequence of the Rv2531c PCR product.
Nucleic acid sequence of the Rv2531c gene is shown. The reverse sequence of the 2844 bp Rv2531c PCR amplified DNA fragment (top strand) is shown aligned against the Rv2531c gene of *M. tuberculosis* (bottom strand). The Rv2531c gene was sequenced using the reverse primer, Rv2531C-REV. This sequence has been matched to the original sequence (Figure 5.1) using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs.

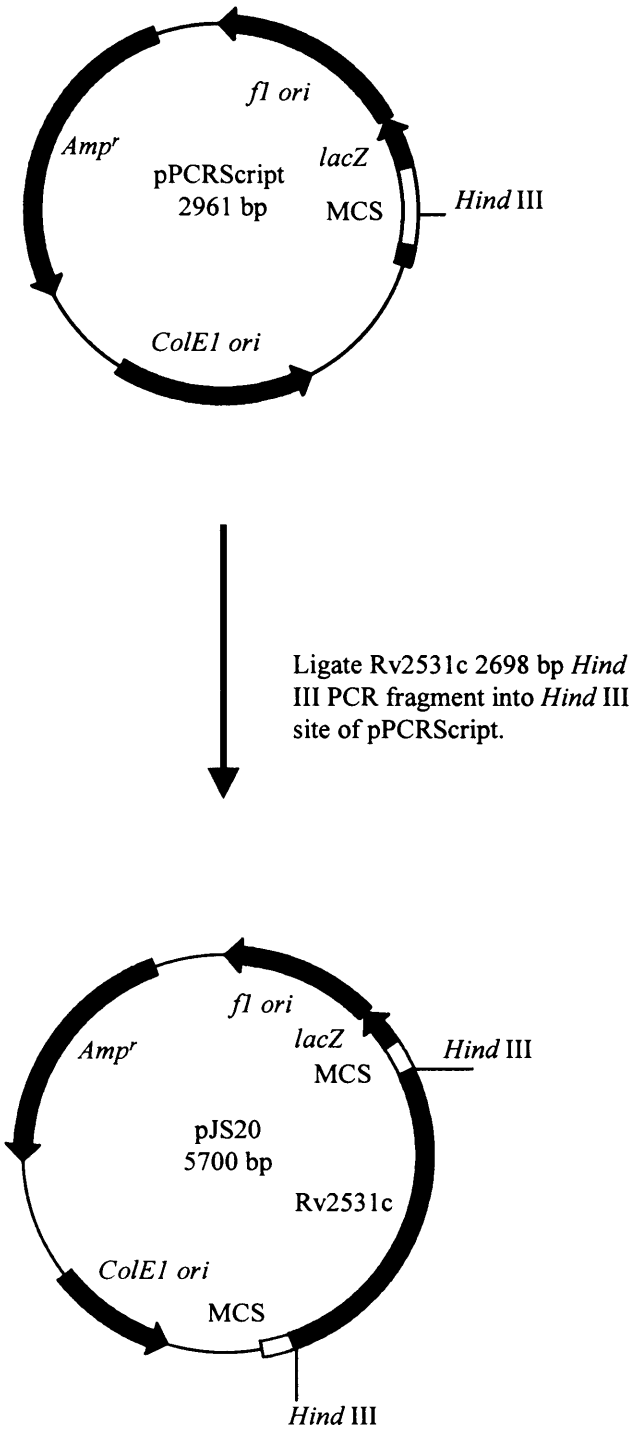


Figure 5.5. Cloning the Rv2531c PCR product into pPCRScript to produce pJS20. The 2698 bp Rv2531c amplified fragment from MTCY159 was digested with *Hind* III and ligated into the *Hind* III site of pPCRScript to produce pJS20. Abbreviations are *Escherichia coli* origin of replication (*ColE1 ori*), ampicillin resistance (*Amp*^{r}), blue/white selection with β-galactosidase (*lacZ*), multiple cloning site (MCS) and f1 filamentous phage origin of replication (*f1 ori*).

μg/ml ampicillin, 0.004% (v/v) X-gal in dimethylformamide and 0.1 mM IPTG. White colonies not expressing β-galactosidase were selected and plasmid DNA was prepared from these transformants.

To confirm the presence of the 2698 bp Rv2531c fragment in pPCRScript, the DNA was digested with *Hind* III or *Eco*R I and run through a 0.7% (w/v) agarose gel. Restriction digest with *Hind* III resulted in two fragments of approximately 2698 bp and 2961 bp and restriction digest with *Eco*R I resulted in one fragment of approximately 6000 bp (Figure 5.6). This demonstrated successful cloning of the Rv2531c fragment into the vector, pPCRScript. This recombinant plasmid was designated pJS20.

5.3.2. Subcloning the Rv2531c fragment into the suicide vector, pSM20 to produce pJS21.

For subsequent subcloning of the DNA fragment of Rv2531c from pJS20 into the suicide vector pSM20 (Selbitschka *et al.*, 1993), the recombinant plasmid pJS20 was digested with *Sca* I and *Hind* III in order to isolate the Rv2531c fragment. Restriction digest with *Sca* I/*Hind* III resulted in three fragments of approximately 2698 bp, 1800 bp and 1200 bp. The 2698 bp Rv2531c fragment was excised from the agarose gel and the DNA isolated using the Sephaglas Bandprep Kit. The Rv2531c DNA was digested with *Hind* III and ligated into the similarly digested and dephosphorylated *Hind* III site of pSM20. The ligation product was introduced into *E. coli* strain DH5α by electroporation and the transformants were selected on ampicillin.

To confirm the presence of the 2698 bp Rv2531c fragment in pSM20, the DNA was digested with *Hind* III or *Eco*R I. Restriction digest with *Hind* III resulted in two fragments of approximately 2698 bp and 4611 bp and restriction digest with *Eco*R I resulted in a one fragment of approximately 7300 bp (Figure 5.7). This demonstrated successful cloning of the Rv2531c fragment into the suicide vector, pSM20. This recombinant plasmid was designated pJS21.

5.3.3. Cloning the kanamycin resistance cassette into pJS21 to produce pJS22.

In order to introduce a selectable marker for the plasmid, the kanamycin cassette from pUC-4K (Pharmacia) was isolated. Plasmid DNA was digested with *Eco*R I resulting in

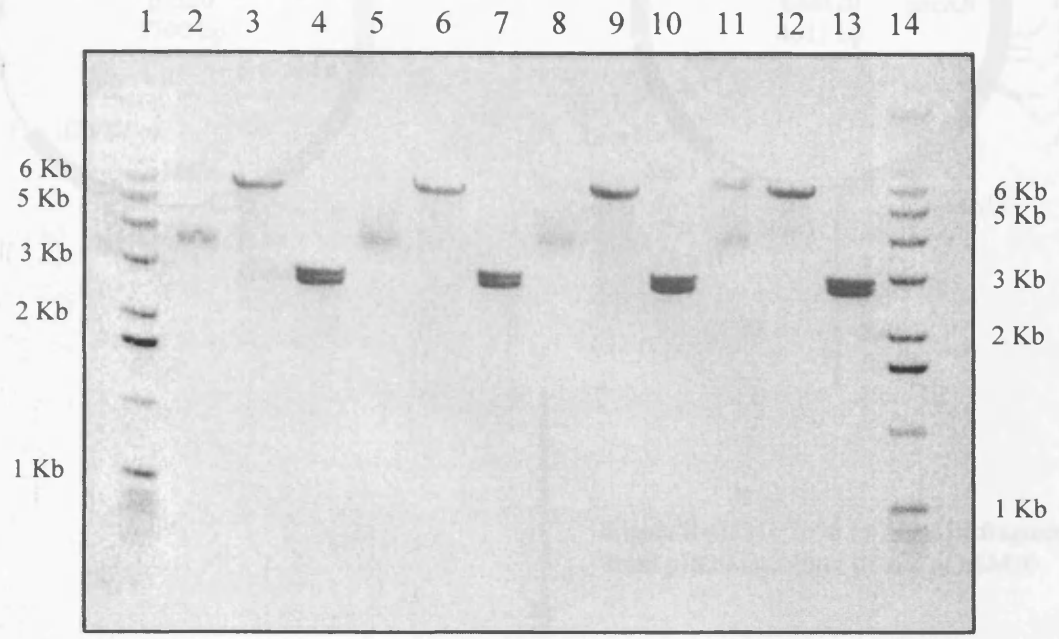


Figure 5.6. Cloning the Rv2531c PCR product into pPCRScript to produce pJS20. To confirm the presence of the 2698 bp Rv2531c fragment in pPCRScript, the DNA was digested with either *Hind* III or *Eco*R I. Restriction digestion with *Hind* III resulted in two fragments of approximately 2698 bp and 2961 bp (Lanes 4, 7, 10 and 13). Restriction digestion with *Eco*R I resulted in one fragment of approximately 6000 bp (Lanes 3, 6, 9 and 12). Lanes 2, 5, 8 and 11 contain undigested DNA. Lanes 1 and 14 contain DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb).

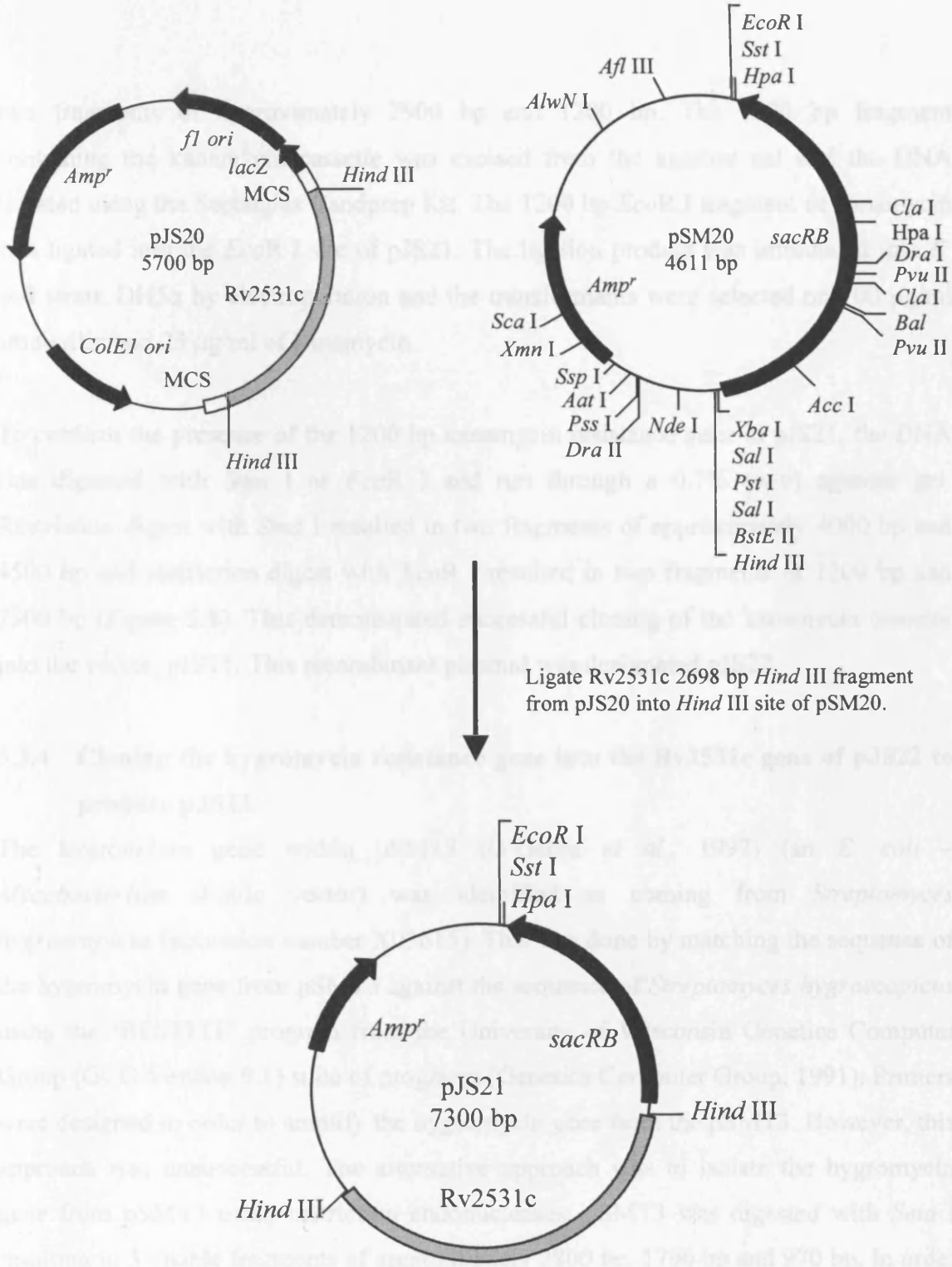


Figure 5.7. Subcloning the Rv2531c fragment into pSM20 to produce pJS21.

The plasmid pJS20 was digested with *Hind* III to remove the 2698 bp Rv2531c gene and ligated into the *Hind* III site of pSM20 to produce pJS21. Abbreviations are putative decarboxylase (Rv2531c), levansucrase - sucrose sensitivity (*sacRB*), *E. coli* origin of replication (*ColE1 ori*), multiple cloning site (MCS), f1 filamentous phage origin of replication (*f1 ori*), blue/white selection with β-galactosidase (*lacZ*) and ampicillin resistance (*Amp*^{r}).

two fragments of approximately 2900 bp and 1200 bp. The 1200 bp fragment containing the kanamycin cassette was excised from the agarose gel and the DNA isolated using the Sephaglas Bandprep Kit. The 1200 bp *Eco*R I fragment of kanamycin was ligated into the *Eco*R I site of pJS21. The ligation product was introduced into *E. coli* strain DH5α by electroporation and the transformants were selected on 100 μg/ml ampicillin and 25 μg/ml of kanamycin.

To confirm the presence of the 1200 bp kanamycin resistance gene in pJS21, the DNA was digested with *Sma* I or *Eco*R I and run through a 0.7% (w/v) agarose gel. Restriction digest with *Sma* I resulted in two fragments of approximately 4000 bp and 4500 bp and restriction digest with *Eco*R I resulted in two fragments of 1200 bp and 7300 bp (Figure 5.8). This demonstrated successful cloning of the kanamycin cassette into the vector, pJS21. This recombinant plasmid was designated pJS22.

5.3.4. Cloning the hygromycin resistance gene into the Rv2531c gene of pJS22 to produce pJS23.

The hygromycin gene within pSMT3 (O'Gaora *et al.*, 1997) (an *E. coli* – *Mycobacterium* shuttle vector) was identified as coming from *Streptomyces hygroscopicus* (accession number X03615). This was done by matching the sequence of the hygromycin gene from pSMT3 against the sequence of *Streptomyces hygroscopicus* using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991). Primers were designed in order to amplify the hygromycin gene from the pSMT3. However, this approach was unsuccessful. The alternative approach was to isolate the hygromycin gene from pSMT3 using restriction endonucleases. pSMT3 was digested with *Sma* I resulting in 3 visible fragments of approximately 2800 bp, 1790 bp and 970 bp. In order to isolate the hygromycin gene, the DNA was further digested with *Pvu* II. This resulted in the digestion of the 2800 bp fragment, into two further fragments of approximately 2200 bp and 600 bp. The 2200 bp *Sma* I/*Pvu* II fragment contained the hygromycin gene. This fragment was excised from an agarose gel and the DNA isolated using the Sephaglas Bandprep Kit. Subsequently, a partial digest of pJS22 DNA was done using *Sma* I in order to linearise the DNA. A partial digest was necessary since two *Sma* I restriction sites were present in the plasmid. The DNA was run through a 0.7% (w/v) agarose gel and the linear fragment of pJS22 DNA was excised and the DNA isolated.

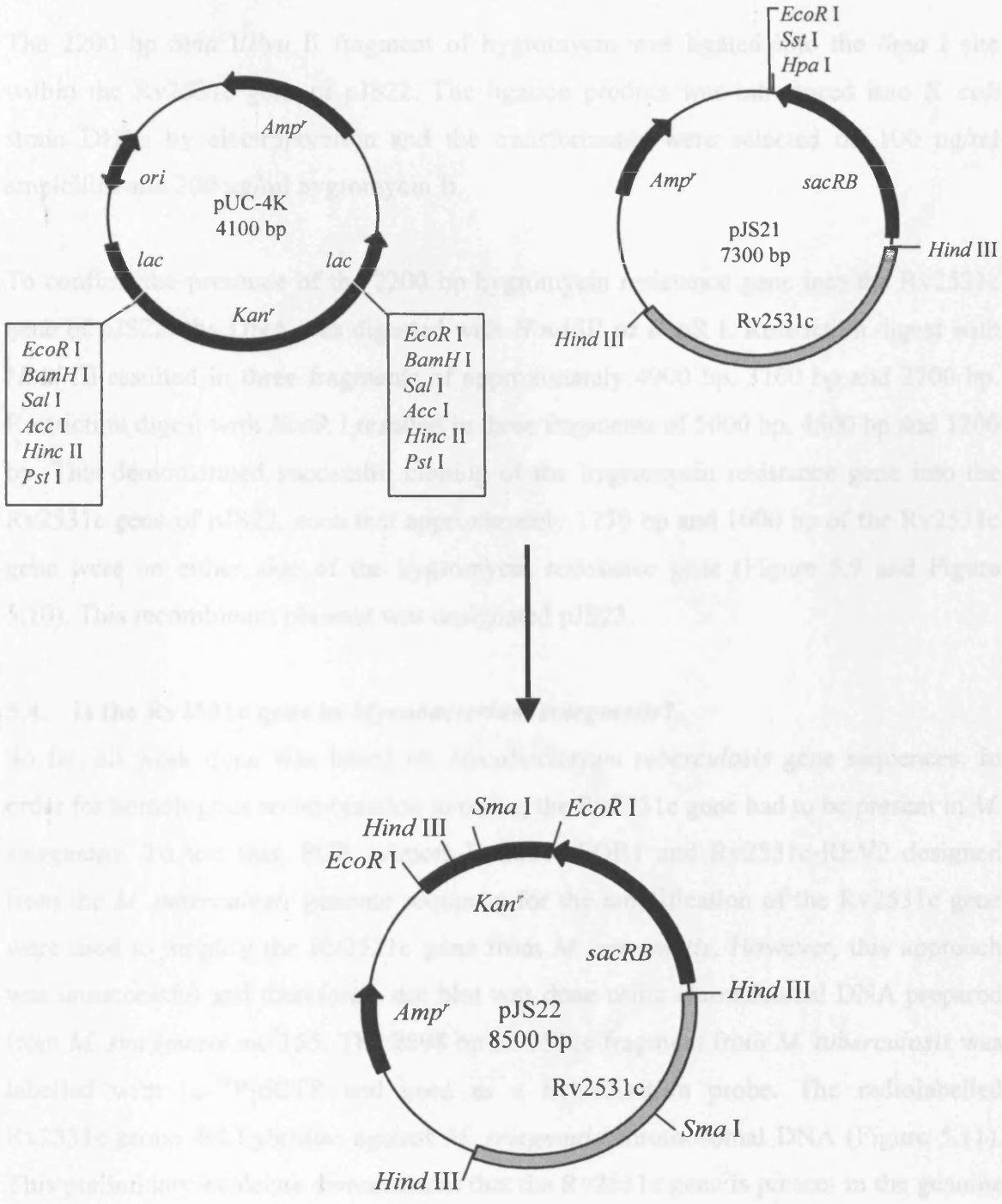


Figure 5.8. Cloning the kanamycin cassette into pJS21 to produce pJS22.

The pUC-4K plasmid carrying the kanamycin cassette was digested with *EcoR* I to remove the kanamycin gene and ligated into the *EcoR* I site of pJS21 to produce pJS22. Abbreviations are putative decarboxylase (Rv2531c), levansucrase - sucrose sensitivity (*sacRB*), *E. coli* origin of replication (*ori*), blue/white selection with β-galactosidase (*lac*), ampicillin resistance (*Amp*^{r}) and kanamycin resistance (*Kan*^{r}).

The 2200 bp *Sma* I/*Pvu* II fragment of hygromycin was ligated into the *Sma* I site within the Rv2531c gene of pJS22. The ligation product was introduced into *E. coli* strain DH5α by electroporation and the transformants were selected on 100 μg/ml ampicillin and 200 μg/ml hygromycin B.

To confirm the presence of the 2200 bp hygromycin resistance gene into the Rv2531c gene of pJS22, the DNA was digested with *Hind* III or *Eco*R I. Restriction digest with *Hind* III resulted in three fragments of approximately 4900 bp, 3100 bp and 2700 bp. Restriction digest with *Eco*R I resulted in three fragments of 5000 bp, 4500 bp and 1200 bp. This demonstrated successful cloning of the hygromycin resistance gene into the Rv2531c gene of pJS22, such that approximately 1770 bp and 1000 bp of the Rv2531c gene were on either side of the hygromycin resistance gene (Figure 5.9 and Figure 5.10). This recombinant plasmid was designated pJS23.

5.4. Is the Rv2531c gene in *Mycobacterium smegmatis*?

So far, all work done was based on *Mycobacterium tuberculosis* gene sequences. In order for homologous recombination to occur, the Rv2531c gene had to be present in *M. smegmatis*. To test this, PCR primers Rv2531c-FOR1 and Rv2531c-REV2 designed from the *M. tuberculosis* genome sequence for the amplification of the Rv2531c gene were used to amplify the Rv2531c gene from *M. smegmatis*. However, this approach was unsuccessful and therefore a dot blot was done using chromosomal DNA prepared from *M. smegmatis* mc^2155. The 2698 bp Rv2531c fragment from *M. tuberculosis* was labelled with [α-^{32}P]dCTP and used as a hybridisation probe. The radiolabelled Rv2531c probe did hybridise against *M. smegmatis* chromosomal DNA (Figure 5.11). This preliminary evidence demonstrates that the Rv2531c gene is present in the genome of *M. smegmatis*.

5.5. Introduction of an Rv2531c mutation in *Mycobacterium smegmatis*.

To introduce a mutation in the chromosomal encoded Rv2531c gene of *M. smegmatis* the following approach was undertaken. The suicide vector that had been constructed, pJS23, encoded the *sacRB* gene; this is a modified derivative of the *Bacillus subtilis sacB* gene, encoding levansucrase that confers sucrose sensitivity, which was used as a selectable marker in *M. smegmatis*. The *Bacillus subtilis sacB* gene has previously been

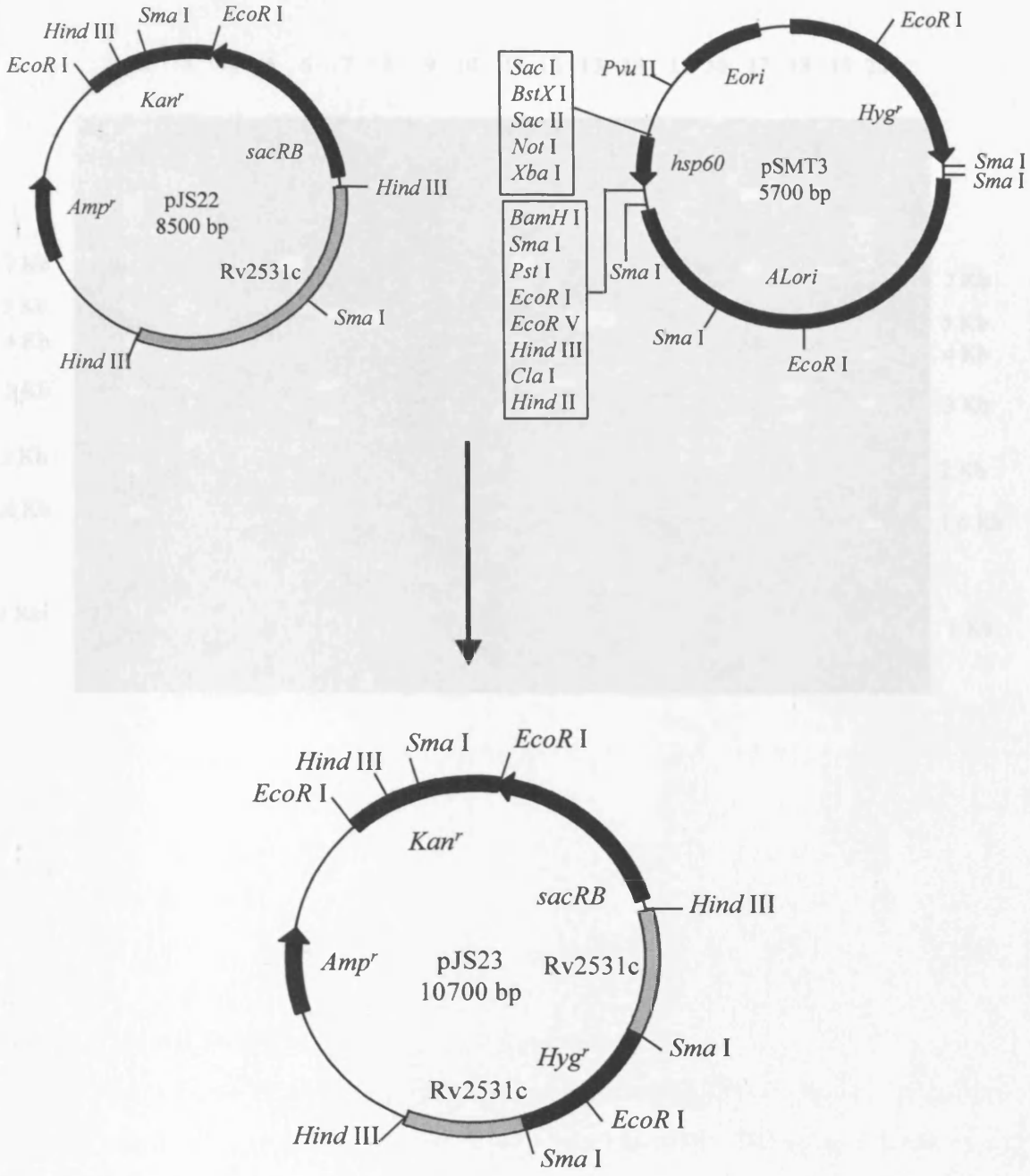


Figure 5.9. Cloning the hygromycin resistance gene into the Rv2531c gene of pJS22 to produce pJS23.

The plasmid pSMT3 was digested with *Sma* I/*Pvu* II to remove the hygromycin gene and ligated into the *Sma* I site of pJS22 to produce pJS23. Abbreviations are putative decarboxylase (Rv2531c), levansucrase - sucrose sensitivity (*sacRB*), ampicillin resistance (*Amp*^{r}), kanamycin resistance (*Kan*^{r}), hygromycin resistance (*Hyg*^{r}), mycobacterial origin of replication (AL*ori*), *E. coli* origin of replication (*Eori*) and *M. bovis* BCG heat shock promoter (*hsp60*).

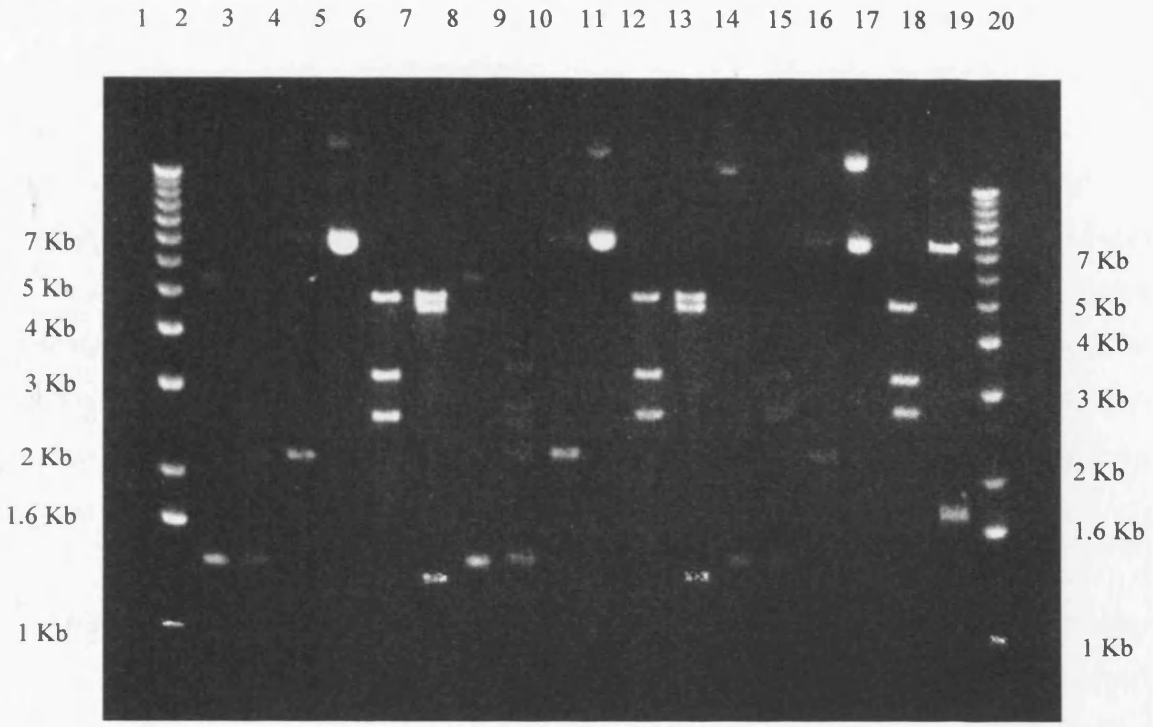
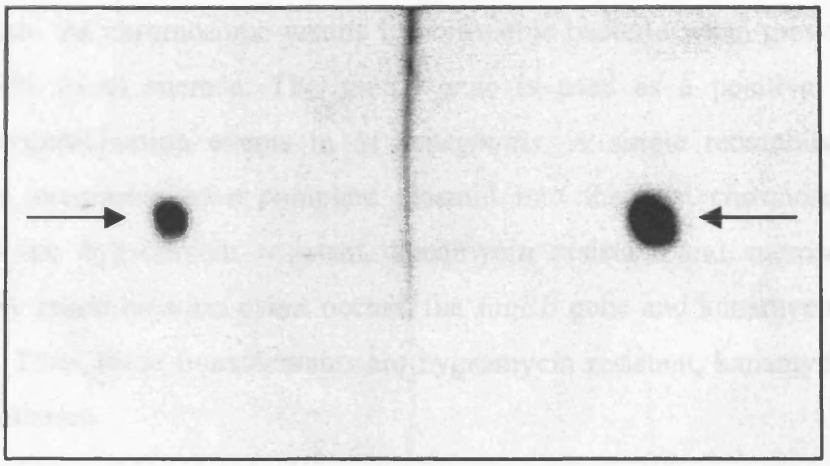


Figure 5.10. Cloning the hygromycin gene into the Rv2531c gene of pJS22 to produce pJS23.

To confirm the presence of the 2200 bp hygromycin resistance gene into the Rv2531c gene of pJS22, the DNA was digested with *Hind* III or *Eco*R I. Restriction digestion with *Hind* III resulted in three fragments of approximately 4900 bp, 3100 bp and 2700 bp (Lanes 6 and 12). Restriction digestion with *Eco*R I resulted in three fragments of 5000 bp, 4500 bp and 1200 bp (Lanes 7 and 13). Lanes 5 and 11 contain undigested DNA. Lanes 1 and 20 contain DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in Kilobases (Kb). All other lanes contain DNA with incorrect fragment sizes.



700 ng 1000 ng

Figure 5.11. Is the Rv2531c gene in *M. smegmatis*?

DNA dot blot of [α-^{32}P]dCTP labelled *M. tuberculosis* Rv2531c probe, hybridised against *M. smegmatis* chromosomal DNA with approximately 700 ng and 1000 ng of DNA bound on the membrane.

demonstrated to confer sucrose sensitivity on mycobacteria and has also been used to generate mutations in mycobacteria (Pelicic *et al.*, 1996a; 1996b). The introduction of a *sacRB* gene into the chromosome results in non-viable bacteria when grown on media containing 10% (w/v) sucrose. The *sacRB* gene is used as a positive marker for selection of recombination events in *M. smegmatis*. A single recombination event results in the integration of a complete plasmid into the host chromosome. These transformants are hygromycin resistant, kanamycin resistant and sucrose sensitive. When a double recombination event occurs, the *sacRB* gene and kanamycin resistance gene are lost. Thus, these transformants are hygromycin resistant, kanamycin sensitive and sucrose resistant.

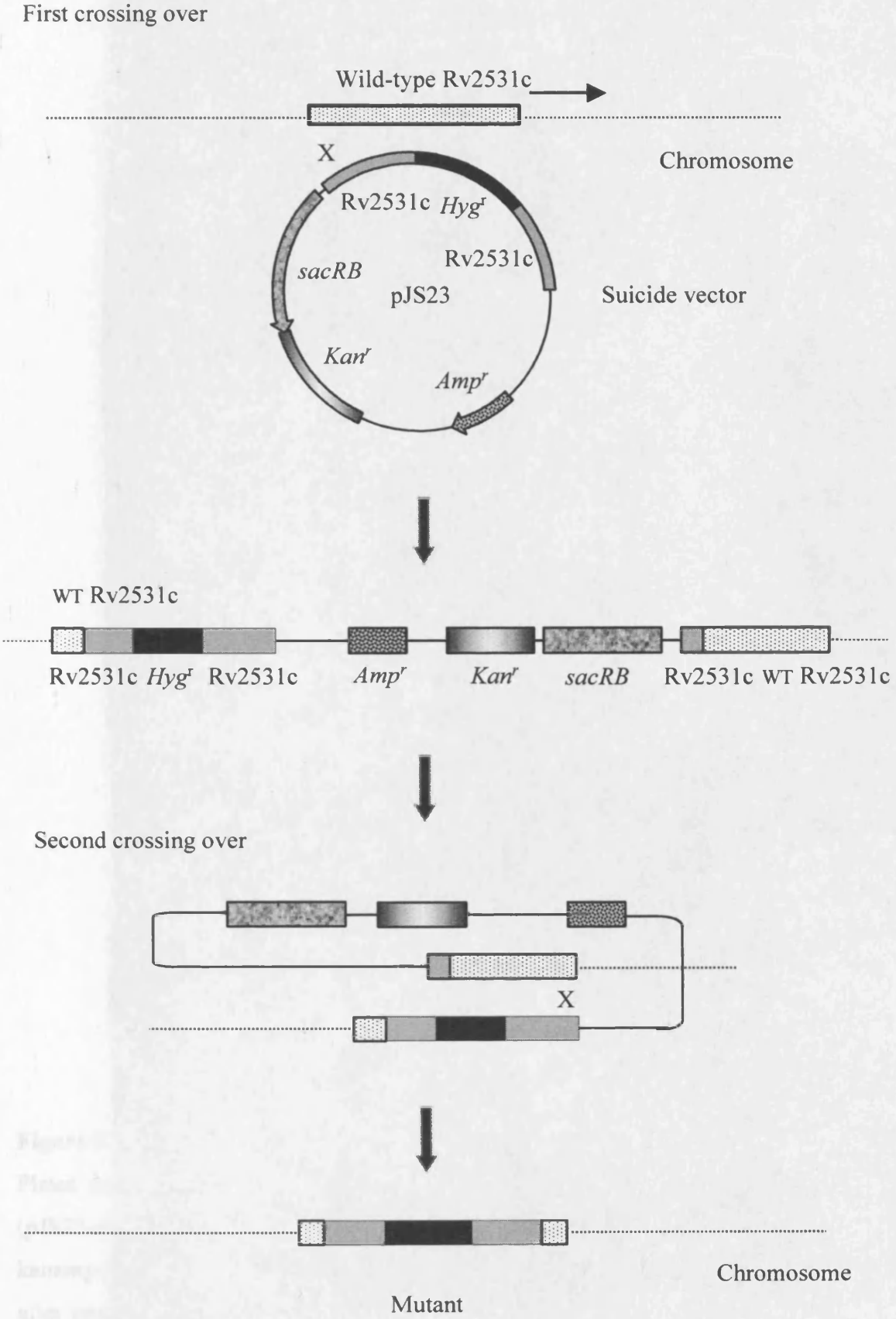
The plasmid DNA, pJS23, was transformed into *M. smegmatis* using electroporation and selected on Luria agar containing 50 μg/ml of hygromycin B. Hygromycin resistant transformants were isolated and replicate plated onto Luria agar containing 25 μg/ml kanamycin or on Luria agar containing 10% (w/v) sucrose, in the absence of sodium chloride. Sodium chloride is not added to the medium because selection of bacteria in the absence of sodium chloride is greater. No growth was observed on kanamycin containing plates, whilst considerable growth was observed on plates containing sucrose suggesting that these transformants no longer encoded kanamycin resistance or *sacRB*. Overall, the transformants isolated, demonstrated hygromycin resistance, kanamycin sensitivity and sucrose resistance; thus, these strains had undergone a double recombination event. One of these recombinants was designated *M. smegmatis* (pJS23-dc) (Figure 5.12 and Figure 5.13).

5.6. PCR to confirm integration of pJS23.

In order to confirm the integration of pJS23 into *M. smegmatis*, a positive PCR for the hygromycin gene was done as described in the materials and methods (section 2.5. and Table 2.3). The forward primer Hyg-F and the reverse primer Hyg-R were designed to amplify 500 bp of the hygromycin gene using genomic DNA isolated from *M. smegmatis* (pJS23-dc). The complete hygromycin gene could not be amplified due to several stem-loops present in the sequence preventing amplification. This finding may also explain why amplification of the whole hygromycin gene from pSMT3 was unsuccessful (section 5.3.4). The hygromycin PCR product from *M. smegmatis* (pJS23-

Figure 5.12. Introduction of an Rv2531c mutation in *M. smegmatis*.

The plasmid, pJS23, was introduced into *M. smegmatis* to allow integration of the plasmid into the host chromosome. A single cross over event was demonstrated by sucrose sensitivity, and kanamycin and hygromycin resistance. A double cross over event was demonstrated by sucrose resistance, kanamycin sensitivity and hygromycin resistance. Abbreviations are putative decarboxylase (Rv2531c), levansucrase - sucrose sensitivity (*sacRB*), ampicillin resistance (*Amp^r*), kanamycin resistance (*Kan^r*) and hygromycin resistance (*Hyg^r*).


First crossing over
Wild-type Rv2531c
X
Chromosome
Rv2531c Hygr
sacRB
Rv2531c
pJS23
Suicide vector
Kanr
Ampr
WT Rv2531c
Rv2531c Hygr Rv2531c
Ampr
Kanr
sacRB
Rv2531c WT Rv2531c
Second crossing over
X
Chromosome
Mutant

Sucrose resistant Hygromycin resistant

Kanamycin sensitive

Figure 5.13. Introduction of an Rv2531c mutation in *M. smegmatis*.

Plates containing Luria agar showing growth or no growth of strain *M. smegmatis* (pJS23-dc). Results demonstrate sucrose resistance, hygromycin resistance and kanamycin sensitivity indicating a double cross-over event occurring in *M. smegmatis* after introduction of the vector pJS23.

dc) was visualised at approximately 500 bp in a 1% (w/v) agarose gel (Figure 5.14). Controls for the PCR included the hygromycin gene that had been isolated from pSMT3 and used to make the construct pJS23, pSMT3 plasmid DNA and *M. smegmatis* mc^2155 genomic DNA. The positive PCR result of the hygromycin gene with *M. smegmatis* (pJS23-dc) but absence of PCR product with *M. smegmatis* mc^2155 confirms that integration of the cassette, pJS23 has occurred.

5.7. Testing for an acid tolerance response.

5.7.1. Testing *M. smegmatis* (pJS23-dc) for an acid tolerance response.

The Rv2531c gene knockout of *M. smegmatis* (pJS23-dc) was used to test whether this gene was involved in the acid tolerance response. The acid tolerance response was tested using hydrochloric acid (HCl) and phosphoric acid (H_3PO_4). An adaptive pH of 4.5 or 4.0 and a lethal pH of 2.5 were used with HCl and an adaptive pH of 5.0 or 4.5 and a lethal pH of 2.5 were used with H_3PO_4. These pHs had been previously identified from the survival of *M. smegmatis* over a range of pH (Chapter 4, section 4.6.1). The pH of the cultures was measured at the end of each experiment using pH indicator strips (Sigma and BDH). At the end of each experiment the pH of the medium was measured, pH readings remained the same ± 0.5 units.

Cultures were grown in 7H9 broth at pH 7.6 and adapted at a pH of 4.5 or pH 4.0 or placed into fresh medium at pH 7.6 and incubated to allow one doubling before cultures were exposed to a lethal pH of 2.5 with HCl as the acidulant (Figure 5.15). Viable counts were measured over six hours. *M. smegmatis* (pJS23-dc) adapted at a pH of 4.5 or pH 4.0 survived significantly ($P< 0.001$) better at pH 2.5 compared with unadapted cultures. For example, percentage viability at the one hour time point was 92% for cultures adapted at pH 4.5, 91% for cultures adapted at pH 4.0 and 25% for unadapted cultures. Furthermore, cultures adapted at pH 4.0 survived significantly ($P< 0.001$) better at pH 2.5 at all time points. However, a reduction in acid tolerance was observed after the four hour time point in both the adapted cultures. Colony forming units for cultures adapted at pH 4.5 were not significantly ($P> 0.05$) different from unadapted cultures at pH 2.5 at six hours. These data suggest that the Rv2531c gene may not be crucial in the acid tolerance response when HCl is the acidulant.

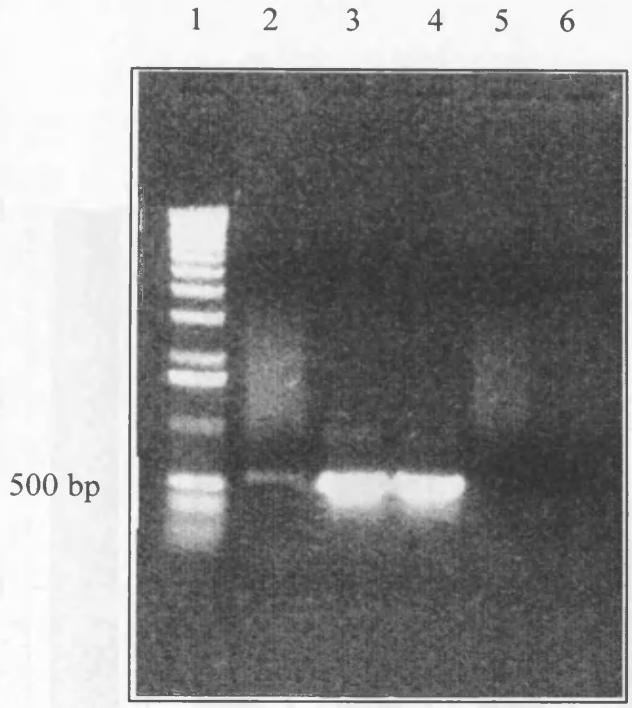


Figure 5.14. Amplification of the hygromycin gene.

Amplification of the hygromycin gene sequence was done using primers Hyg-F and Hyg-R. Lane 1 contains DNA size marker (1 Kb ladder, Gibco-BRL), with fragment sizes shown in basepairs (bp). Lane 2 contains amplified product from *M. smegmatis* (pJS23-dc) at approximately 500 bp. Lane 3 contains amplified product from the hygromycin gene isolated from pSMT3 and lane 4 contains amplified product from pSMT3 plasmid DNA. Lane 5 contains *M. smegmatis* mc^2155 genomic DNA, no fragment was amplified. Lanes 3 to 4 show a PCR product at approximately 500 bp. Lane 6 contains the negative control.

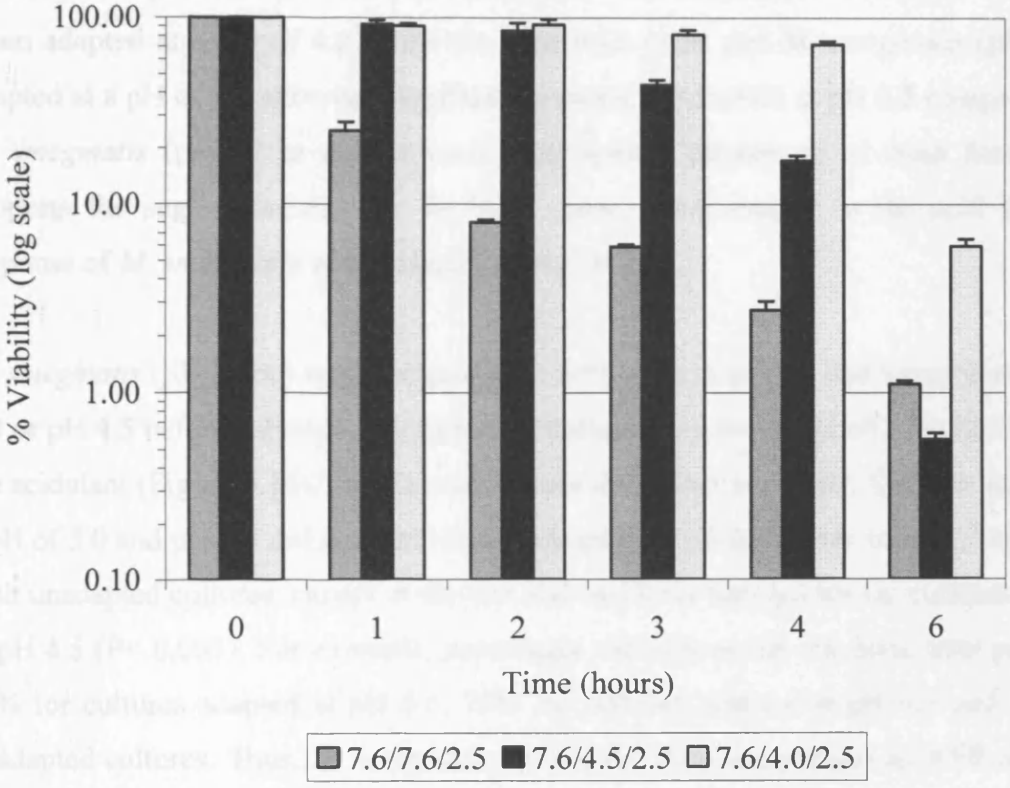


Figure 5.15. Testing *M. smegmatis* (pJS23-dc) for an ATR with hydrochloric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 4.5 or pH 4.0 for four hours or grown at pH 7.6 for two hours before exposure to a lethal pH of 2.5. The graph shows percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS23-dc) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with hydrochloric acid (HCl). Log cfu/ml mean values for cultures grown at pH 7.6, 4.5 or 4.0 at 0 time are 8.42, 8.37 and 8.29, respectively.

When the data from *M. smegmatis* (pJS23-dc) (Figure 5.15) was compared to the data from *M. smegmatis* (pJS12) (wild-type) after HCl (Figure 4.24) it is seen that the survival of *M. smegmatis* (pJS23-dc) at pH 2.5 was not less than the that of *M. smegmatis* (pJS12) during the first four hours after adaptation at either pH 4.5 or pH 4.0. No significant ($P > 0.05$) difference at pH 2.5 was found between the two strains. In addition, no significant ($P > 0.05$) difference was seen at pH 2.5 between the two strains when adapted at a pH of 4.0 at the six hour time point and *M. smegmatis* (pJS23-dc) adapted at a pH of 4.5 survived significantly worse ($P < 0.001$) at pH 2.5 compared with *M. smegmatis* (pJS12) at the six hour time point. Comparison of these data further supports the suggestion that the Rv2531c gene is not crucial to the acid tolerance response of *M. smegmatis* when adapted using HCl.

M. smegmatis (pJS23-dc) was also grown in 7H9 broth at pH 7.6 and adapted at a pH of 5.0 or pH 4.5 before subsequently exposing cultures to a lethal pH of 2.5 with H_3PO_4 as the acidulant (Figure 5.16). Viable counts were done over six hours. Cultures adapted at a pH of 5.0 and pH 4.5 did not survive significantly ($P > 0.05$) better at pH 2.5 compared with unadapted cultures, except at the one and two hour time points for cultures adapted at pH 4.5 ($P < 0.001$). For example, percentage viability at the one hour time point was 72% for cultures adapted at pH 5.0, 74% for cultures adapted at pH 4.5 and 34% for unadapted cultures. Thus, *M. smegmatis* (pJS23-dc) does not possess an ATR at pH 2.5 when adapted at pH 4.5 or pH 5.0 using H_3PO_4. These data suggest that the Rv2531c gene is involved in the acid tolerance response when H_3PO_4 is the acidulant.

When the data from *M. smegmatis* (pJS23-dc) (Figure 5.16) was compared to the data from *M. smegmatis* (pJS12) (wild-type) after H_3PO_4 (Figure 4.23.,) it is seen that the survival of *M. smegmatis* (pJS23-dc) at pH 2.5 was not less than that of *M. smegmatis*:pJS12 after adaptation at pH 5.0. No significant ($P > 0.05$) difference at pH 2.5 was found between the two strains. In addition, a significant ($P < 0.001$) difference was seen at pH 2.5 between the two strains when adapted at a pH of 4.5 and *M. smegmatis* (pJS23-dc) adapted at a pH of 4.5 survived significantly ($P < 0.001$) worse at pH 2.5 compared with *M. smegmatis* (pJS12) at and after the three hour time point. For example, percentage viability at the three hour time point was 2% for *M. smegmatis* (pJS23-dc) and 12% for *M. smegmatis* (pJS12). Thus, under these conditions, these data

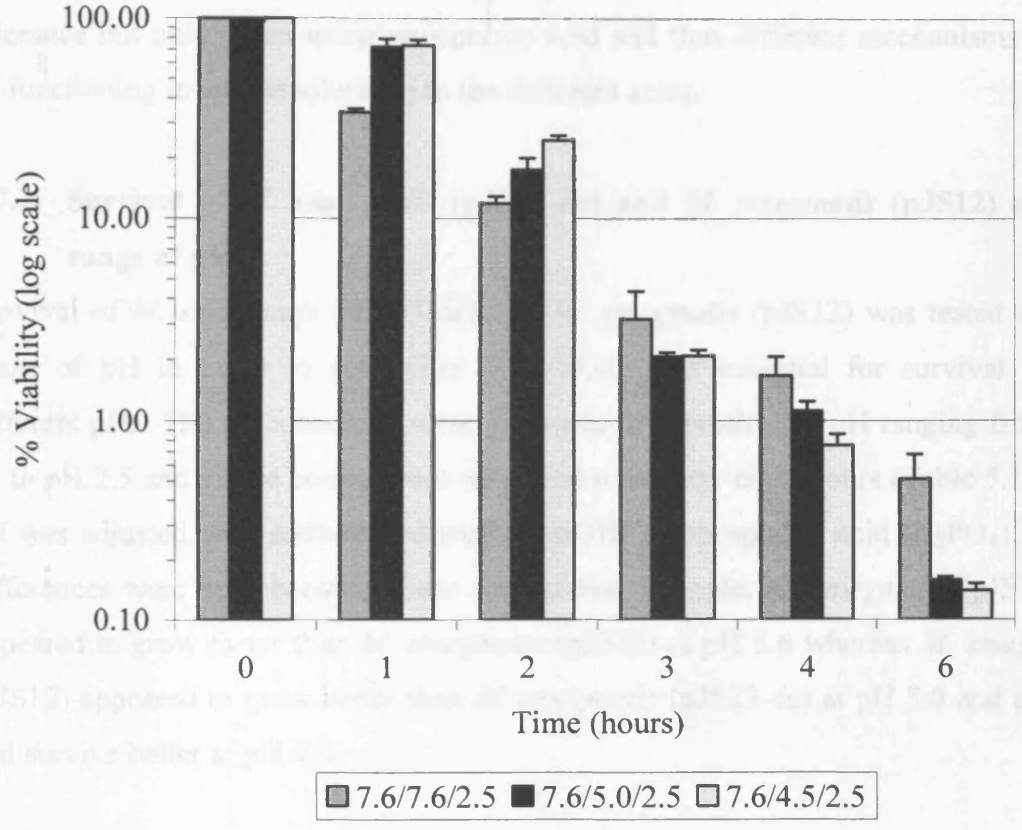


Figure 5.16. Testing *M. smegmatis* (pJS23-dc) for an ATR with phosphoric acid as the acidulant.

Cultures were grown at pH 7.6 and adapted at pH 5.0 or pH 4.5 for four hours or grown at pH 7.6 for two hours before exposure at a lethal pH of 2.5. The graph shows percentage viability (log scale) at the lethal pH for *M. smegmatis* (pJS23-dc) over 6 hours. All values represent the mean ± S.E.M. of four experiments. Cultures were acidified with phosphoric acid (H_3PO_4). Log cfu/ml mean values for cultures grown at pH 7.6, 5.5 or 4.5 at 0 time are 8.36, 8.26 and 8.30, respectively.

suggest that the Rv2531c gene was involved in the acid tolerance response when using H_3PO_4 as the acidulant. Overall, when the response of the two strains *M. smegmatis* (pJS23-dc) (Rv2531c gene knockout) and *M. smegmatis* (pJS12) (wild-type) were compared, acid tolerance by *M. smegmatis* (pJS23-dc) was not exhibited with phosphoric acid as the acidulant but was observed with *M. smegmatis* (pJS23-dc) in hydrochloric acid. These data suggest that the Rv2531c gene is involved in acid tolerance but only when using phosphoric acid and thus different mechanisms could be functioning to enable tolerance to the different acids.

5.7.2. Survival of *M. smegmatis* (pJS23-dc) and *M. smegmatis* (pJS12) over a range of pH.

Survival of *M. smegmatis* (pJS23-dc) and *M. smegmatis* (pJS12) was tested over a range of pH in order to determine if Rv2531c was essential for survival at the different pHs. The mycobacteria were grown in 7H9 broth with pH ranging from pH 10 to pH 2.5 and viable counts were measured over forty eight hours (Table 5.1). The pH was adjusted with sodium hydroxide (NaOH) or phosphoric acid (H_3PO_4). Some differences were seen between these strains. For example, *M. smegmatis* (pJS23-dc) appeared to grow faster than *M. smegmatis* (pJS12) at pH 8.6 whereas *M. smegmatis* (pJS12) appeared to grow better than *M. smegmatis* (pJS23-dc) at pH 5.0 and pH 4.5 and survive better at pH 4.0.

M. smegmatis:pJS12

Time (hrs)	0	6	24	32	48
pH					
10	7.01	7.14	6.93	6.95	6.46
9.5	7.00	7.02	6.78	6.95	7.83
9	7.00	7.11	6.78	8.17	9.14
8.6	6.99	7.19	6.97	8.26	9.38
8	6.98	7.37	8.65	9.02	9.46
7.6	6.97	7.42	8.77	9.10	9.42
6	7.00	7.34	8.91	8.85	9.34
5	7.02	7.30	8.86	9.07	9.23
4.5	6.98	7.08	7.34	7.71	7.54
4	7.06	7.06	6.54	6.53	6.13
3.5	7.08	6.51	4.51	4.60	4.68
3	6.99	3.43	4.08	2.48	4.00
2.5	6.88	3.46	3.91	2.48	4.10

M. smegmatis:pJS23-dc

Time (hrs)	0	6	24	32	48
pH					
10	7.12	7.02	6.89	6.73	6.35
9.5	6.88	6.93	6.80	7.09	7.66
9	7.04	6.85	7.23	7.74	9.02
8.6	7.03	7.01	8.37	8.81	9.41
8	7.03	7.33	8.98	9.20	9.44
7.6	7.01	7.50	8.75	9.24	9.36
6	7.01	7.40	8.93	9.05	9.25
5	6.99	6.14	8.05	8.53	8.21
4.5	6.94	6.88	6.82	6.92	6.53
4	6.98	6.73	6.23	6.15	5.49
3.5	7.01	6.66	4.66	4.20	4.12
3	6.95	3.72	4.92	2.30	3.04
2.5	6.95	2.48	3.08	2.70	4.08

Table 5.1. Survival of *M. smegmatis* (pJS23-dc) and *M. smegmatis* (pJS12) over a range of pH adjusted with phosphoric acid or sodium hydroxide.

Cultures of *M. smegmatis* (pJS23-dc) and *M. smegmatis* (pJS12) were grown at pH ranging from pH 10 to pH 2.5. Results show a comparison of the log colony forming units/ml for *M. smegmatis* (pJS23-dc) and *M. smegmatis* (pJS12) over 48 hours. All values within each experiment are the mean of two counts taken from a single sample at the appropriate time point.

CHAPTER 6: DISCUSSION.

6.1. The *lux* genes.

The aim of this work was to construct an expression vector based on the *Photorhabdus* (*Xenorhabdus*) *luminescens lux* operon, *luxCDABE*, for use as a reporter system in order to develop a method for the rapid testing of antimycobacterial drugs. The expression vector pJS6 was constructed for this purpose. Initial work was done using *M. smegmatis* and *M. aurum* because these organisms were not pathogenic and therefore did not require high containment facilities and also they were rapidly growing mycobacteria and thus allowed the experiments to be done frequently. Also *M. aurum* was used for screening because its sensitivity is comparable to *M. tuberculosis*. Subsequently, work was done using *M. bovis* BCG and *M. tuberculosis*.

The majority of studies have employed *lux* genes from *Vibrio fischeri* and *Vibrio harveyi* and most commonly only the *luxAB* genes have been used. *Vibrio fischeri* and *V. harveyi lux* genes have been expressed in a number of species, including Gram-positive bacteria, Gram-negative bacteria, and plant and mammalian pathogens. For example, *Bacillus subtilis*, *Escherichia coli*, *Agrobacterium rhizogenes* and *Salmonella typhimurium* (Baldwin *et al.*, 1984; Shaw and Kado, 1986; Carmi *et al.*, 1987; Jassim *et al.*, 1990). More recently work has been done using *P. luminescens lux* genes.

To allow measurement of bacteria and to eliminate the necessity for the addition of aldehyde, the entire *lux* operon from *P. luminescens*, *luxCDABE*, was employed for the construction of the expression vector pJS6. Addition of aldehyde to bacteria was not required for light production if the genes responsible for aldehyde synthesis (*luxCDE*) were also transferred. The *lux* operon from *P. luminescens* was used for a number of reasons; the *luxAB* genes that encode luciferase are able to provide high responses to aldehyde (Schmidt *et al.*, 1989; Meighen, 1991), although this is also true of *V. harveyi* (Meighen, 1991). In addition, the *lux* system derived from *P. luminescens* is stable at higher temperatures than the *V. fischeri lux* system. Luciferase derived from *V. fischeri* has a limited temperature range, it only functions appropriately at temperatures below 30°C and is unstable at higher temperatures. *P. luminescens* luciferase is stable at 37°C and is stable for more than three hours at 45°C when compared to five minutes at 45°C for *V. harveyi* luciferase (Meighen, 1991). However, *V. harveyi* luciferase is also stable up to 37°C. Excision of the *lux* operon of *P. luminescens* from pSfi390 was easier than using the *V. harveyi luxCDABE* genes because of the presence of numerous common

restriction sites within the *lux* operon and the lack of unique restriction sites (Meighen, 1991). Also, *P. luminescens luxCDABE* operon has previously been used in our laboratory and was shown to be bioluminescent and stable in *E. coli* (Griffiths, 2000). Also, in the bacterial bioluminescence reaction, a key intermediate 4a-hydroperoxy-FMN (designated II) is formed by reacting luciferase bound $FMNH_2$ with O_2. This immediate although stable in *V. harveyi* was found to be more highly stable in *P. luminescens* (Xi *et al.*, 1991). The high stability of the luciferase intermediate II, high production of light and stability of the *lux* operon from *P. luminescens* in *E. coli* made this *lux* operon most suitable for use as a reporter system, although *V. harveyi* also could have been suitable for use.

A highly bioluminescent phenotype was detected in *E. coli* containing the expression vector pJS6 and this was the only organism in which the vector was found to be stable. Unfortunately, no light was detected in *M. smegmatis* or *M. aurum* containing pJS6. Interestingly, the entire *lux* operon was found to function only in the slow growing mycobacteria. Light was detected in BCG and *M. tuberculosis* but pJS6 was found to be unstable in both mycobacteria. This phenomenon could have been due to several reasons. Aldehyde sensitivity has been described as a possible reason. Increased concentrations of aldehyde inhibit the activity of luciferase and this has been reported for *V. harveyi* luciferase (Hastings *et al.*, 1969) and for *P. luminescens* Hw luciferase (Xi *et al.*, 1991). Further experiments were done to determine whether the continual production of aldehyde through the constitutive expression of the *lux* operon was causing cell toxicity and loss of light production. A vector was constructed using only the *luxAB* genes from *P. luminescens*, to generate pSP1. Again, no light was detected when decanal was added, from *M. smegmatis* transformed with pSP1. This indicated that the production of aldehyde from *luxCDE* genes in *M. smegmatis* might not be the cause of cell toxicity since light was not detected even in the absence of these genes.

A recent study has demonstrated that light production could be aldehyde-limiting rather than inhibition caused by overproduction of aldehyde. The *luxCDABE* operon from *Photobacterium leiognathi* was cloned and transferred into the genome of *Anabaena* sp., (Gram-negative *cyanobacterium*). Colonies with limited production of aldehyde showed poor or no expression of bioluminescence (Fernández-Pinas and Wolk, 1994). The addition of exogenous aldehyde to these colonies containing *luxCDABE* resulted in

increased levels of bioluminescence, thus demonstrating aldehyde-limited bioluminescence. In this work, the addition of exogenous aldehyde to *M. smegmatis* containing *luxCDABE* did not result in light production. Also, no toxicity was observed in *Anabaena* sp., over prolonged periods of bioluminescence. Interestingly, colonies of *Anabaena* containing the complete *lux* operon and that were highly bioluminescent exhibited a reduction in bioluminescence when supplemented with exogenous aldehyde, demonstrating aldehyde toxicity (Fernández-Pinas and Wolk, 1994).

The variation in the expression of *lux* and in the instability of the constructs may possibly have resulted due to read-through. Expression of luciferase occurred with both expression vectors, *E. coli* (pJS5) and *E. coli* (pJS6). Since *luxCDABE* was orientated in the opposite direction to the *hsp60* promoter in *E. coli* (pJS5), expression therefore occurred independently of the promoter. The results consequently suggest the occurrence of read-through leading to the expression of luciferase. It could be argued as to whether the *hsp60* promoter was actually functional in *E. coli* (pJS6) due to the possibility of read-through. No light was detected from *M. smegmatis* and *M. aurum* when transformed with the expression vector pJS6, whilst expression of *lux* occurred in *M. bovis* BCG and *M. tuberculosis* although unstable. It could have been possible that read-through led to the expression of *luxCDABE* in *M. bovis* BCG and *M. tuberculosis*. The instability of the constructs could have been due the transcription of an antisense RNA strand that formed duplexes with the sense RNA strand and resulted in the obstruction of translation. In *E. coli* (pJS6) read-through may not have been a problem given that the expression of *lux* was stable. A number of constructs however, have been generated using the *Escherichia coli-Mycobacterium* shuttle vector pSMT3. The *luxAB* genes from *V. harveyi* and *P. luminescens* have been cloned into pSMT3 and transformed into *M. smegmatis*, *M. aurum* or *M. tuberculosis*. All constructs expressed luciferase and were stable (Snewin *et al.*, 1999). In order to clarify the situation, the addition of a terminator sequence downstream of the *lux* gene would prevent any possible read-through from occurring.

Light was detected from *M. smegmatis* containing the *luxAB* genes from *V. harveyi* and from *M. aurum* transformed with the *luxAB* genes from *P. luminescens* and *V. harveyi*. There are no apparent differences between the *lux* genes from *P. luminescens* and *V. harveyi* that explain differences in expression. The bioluminescence system of *P.*

luminescens is similar to that of marine bacteria, as demonstrated by studies that show that the subunits of *P. luminescens* could interact with the subunits of *V. harveyi* to form an active hybrid luciferase (Schmidt *et al.*, 1989; Szittner and Meighen, 1993). The α-subunit of *V. harveyi* combined with the β-subunit of *P. luminescens* yielded three-fold higher luciferase activity compared to any other combination, including subunits from the same species (Schmidt *et al.*, 1989). Also, the luciferase of *P. luminescens* and *V. harveyi* were found to have the highest homology between any two species with 84% identity in the α-subunit and 59% identity in the β-subunit (Johnston *et al.*, 1990; Frackman *et al.*, 1990).

A recent study has compared luciferase from *P. luminescens* and *V. harveyi* and the results were found to be contradictory to the results in this thesis. The *luxAB* genes from *P. luminescens* and *V. harveyi* were cloned into an *Escherichia coli-Mycobacterium* shuttle vector, under control of the BCG *hsp*60 promoter to generate pSMT5 and pSMT1 respectively (Snewin *et al.*, 1999). The constructs were subsequently transformed into *M. smegmatis* and bioluminescence was detected in the recombinant mycobacteria containing both these constructs (Snewin *et al.*, 1999). In this work, no light was detected from *M. smegmatis* containing *luxAB* from *P. luminescens*, although the same vector was used for the basis of construction. The only differences that could be found between these two constructs were *M. smegmatis* 1-2c (an electrocompetent derivative of mc^26) and *P. luminescens* strain Hm was employed compared to *M. smegmatis* mc^2155 and *P. luminescens* strain Hb used in this work. In addition, expression of the complete *lux* operon was found to be unstable in mycobacteria (Snewin *et al.*, 1999). This finding supports the work done in this thesis, the *lux* operon from *P. luminescens* was also found to be unstable in mycobacteria. No indication of the amount of light produced by the unstable recombinant mycobacteria was given or the species of *Mycobacterium* employed.

P. luminescens luxCDABE has been used successfully by other groups in a number of bacteria, other than mycobacteria (Purdy and Park, 1993; Marincs and White, 1994; Forde *et al.*, 1998; Winson *et al.*, 1998; Salisbury *et al.*, 1999). In one study, plasmid and mini-Tn5 vectors containing the *luxCDABE* cassette derived from *P. luminescens* was constructed for use in Gram-negative bacteria (Winson *et al.*, 1998). These bacteria included *Escherichia coli*, *Aeromonas hydrophila* and *Chromobacterium violaceum*.

Plasmids containing the *luxCDABE* genes and subsequently introduced into *E. coli* yielded a highly bioluminescent phenotype (Winson *et al.*, 1998). In this work, high levels of light were also generated from *E. coli* transformants containing *luxCDABE* from *P. luminescens* and these transformants were stable. Using the plasmid and mini-Tn5 constructs containing the *lux* cassette, mutants were identified in *C. violaceum* and *A. hydrophila*. This study demonstrated that *luxCDABE* from *P. luminescens* could be used as a reporter of gene expression (Winson *et al.*, 1998).

To elucidate why bioluminescence was not detected or why cells had become dark, gene transcription was analysed in *M. smegmatis* containing *P. luminescens luxCDABE*. In *M. smegmatis* (pJS6), no transcription was seen in *luxAB* encoding luciferase and in the first gene in the operon, *luxC*, encoding aldehyde synthesis. Since no luciferase was produced, no light would be detected in *M. smegmatis* (pJS6) even with the addition of decanal. Firstly, there is a possibility that generation of duplex RNA followed by its rapid degradation resulted in this finding. Secondly, the marine and terrestrial bioluminescent bacteria from which the *lux* genes have been identified are all Gram-negative bacteria. It is possible that the transcriptional machinery of Gram-positive organisms do not recognise the genes from a Gram-negative organism and thus subsequently does not lead to light production. Studies have shown that when luciferase genes from a Gram-negative bacterium are expressed in a Gram-positive bacterium, such as *B. subtilis*, this resulted in light production that was 100-fold lower compared to the optimum light production from a Gram-negative bacterium (Karp, 1989; Jacobs *et al.*, 1991). Also, *E. coli* containing the *luxAB* genes from *V. harveyi* emitted light rapidly and this light remained constant or rose as culture continued to grow. In *B. subtilis* the addition of aldehyde resulted in a rapid response but this decayed quickly (Karp, 1989). It has been suggested that Gram-positive bacteria have difficulties in obtaining high levels of bioluminescence because they have an insufficient capacity to produce and/or regenerate reduced flavin mononucleotide for the bacterial *lux* reaction (Karp, 1989).

A recent study has demonstrated that the introduction of the *lux* operon from *P. luminescens* or the *lux* operon from other Gram-negative bioluminescent bacteria into Gram-positive bacteria results in little or no light, despite the strength of the promoter proceeding this operon (Francis *et al.*, 2000). It has been reported that Gram-positive

ribosomes are unable to translate mRNAs containing weak Gram-negative ribosomal-binding sites (Vallanoweth and Rabinowitz, 1992). Thus, in order to generate stable constructs for use particularly in Gram-positive bacteria, each gene (*lux*A, -B, -C, -D, and -E) of the *P. luminescens lux* operon was modified by replacing the Gram-negative ribosome-binding site with the Gram-positive ribosome-binding site upstream of the start codon of each gene (Francis *et al.*, 2000). The introduction of this construct into *Staphylococcus aureus* (Gram-positive) resulted in highly bioluminescent transformants that were stable at 37°C and did not require exogenous aldehyde. *S. aureus* containing the unmodified *luxCDABE* operon (which contained a Gram-negative ribosome-binding site) was shown not to be bioluminescent (Francis *et al.*, 2000).

A further study has demonstrated that it is possible to generate bioluminescent Gram-positive bacteria (Jacobs *et al.*, 1991). The fused *luxAB* gene of *V. harveyi* was shown to generate a highly bioluminescent phenotype in *B. subtilis* containing a strong promoter upstream of *luxAB* compared to the unfused *luxAB* genes. This translational fusion resulted in light production from *B. subtilis* that was approximately four-fold greater than the fused *luxAB* gene (pSB252) in *E. coli* (Hill *et al.*, 1991; Jacobs *et al.*, 1991). In *E. coli* the unfused *luxAB* (pSB230) products produce higher bioluminescence than pSB252 (Hill *et al.*, 1991).

It would be of interest to see whether the modified construct containing a Gram-positive ribosome-binding site upstream of the start codon of each Gram-negative gene of *P. luminescens* would work in mycobacteria and whether these recombinant mycobacteria could generate high stable levels of light. Also, whether translational fusion of the *luxCDABE* genes is possible for subsequent use in Gram-positive bacteria.

6.2. Alamar blue assay verses the bioluminescence assay.

In order to assay for antimycobacterial activity of the thousands of compounds generated by modern methods of combinatorial chemistry a rapid high-throughput screen is required. The work in this thesis compared the effectiveness of two assays, the alamar blue and bioluminescent assay for the rapid estimation of antimycobacterial activity of drugs against *M. aurum* and *M. smegmatis*. Both assays showed advantages and disadvantages but overall, the bacterial *lux*-based bioluminescent assay was judged

to offer the greatest potential for a simple, rapid, cost-effective and non-radioactive assay for use in high-throughput screening of new antimycobacterial agents.

There are several drugs that are available for use to treat tuberculosis; this study looks at only a small fraction of them. They are important chemotherapeutic agents used to treat infections caused by strains of *Mycobacterium*. Rifampicin and pyrazinamide are both considered as "firstline" drugs, amikacin as a "secondline" drug. A vast number of drugs do not have to be tested in order to compare the validity of the two assays. These anti-tuberculosis drugs are considered to be important and therefore have been chosen.

In terms of the time-scale for the methods, results were obtained in forty eight hours for the alamar blue assay while only twenty four hours was needed for the bioluminescent assay. Hence, a greater number of compounds could be tested using the bioluminescent assay. A further disadvantage of the alamar blue assay is that microbial contaminants are also able to reduce the dye and this could result in false positives and incorrect interpretation of the data. In the bioluminescence assay only recombinant bacteria containing the *lux* genes are expected to produce bioluminescence and thus the opportunity for inaccurate data is unlikely to be a result of contamination.

Using alamar blue in the detection of antimycobacterial activity, dye reduction is seen as a shift in colour that can be measured by simple visual inspection without the need for expensive equipment. For high-throughput screening visual reading of the alamar blue assay would not be satisfactory. Quantifying the results, however, would be useful in interpreting the findings, especially when varying tones of colour are produced. However, the quantitative analysis for *M. aurum* by spectrophotometry method was haphazard and therefore the method is only qualitative. In contrast, the bioluminescent assay generated reproducible results using the luminoskan luminometer. Another advantage of the bioluminescent assay was the clear-cut differences between a negative and positive result. In contrast, there was little difference between the fluorimeter readings of the pink and blue wells of the alamar blue assay. Even the visual assessment of the alamar blue assay can be impaired by the light sensitivity of the dye, which results in faint test wells that make assessment difficult.

There was little variation in rifampicin or pyrazinamide minimum light inhibition (MLI) and minimum reduction change (MRC) with the bioluminescent or visually read alamar blue assays respectively. Rifampicin inhibited *M. smegmatis* and *M. aurum* when tested using the bioluminescent assay. Rifampicin is an inhibitor of bacterial DNA-dependent RNA polymerase. In the bioluminescent assay, rifampicin inhibits the transcription of the *lux* genes by interacting with the RNA polymerase and thereby leads to a reduced expression of *lux* as assayed by a reduction in bioluminescence.

Pyrazinamide had no effect on *M. smegmatis* or *M. aurum* when tested using the bioluminescent assay. Pyrazinamide is hydrolysed by pyrazinamidase (PZase) to its active form, pyrazinoic acid. Under conditions of external acidic pH, pyrazinoic acid accumulates in the cell and results in cell susceptibility. *M. smegmatis* however, possesses two highly active PZases and a highly active efflux pump that allows the conversion of pyrazinamide to pyrazinoic acid very rapidly and the expulsion of pyrazinoic acid from the cell (Zhang *et al.*, 1999). Thus, resulting in the resistance of *M. smegmatis* to pyrazinamide. This could also be occurring with *M. aurum*.

The observations from the study nevertheless suggested that the bioluminescent assay had greater sensitivity for the detection of antimycobacterial activity because with *M. aurum*, amikacin was determined to have a MRC of 250 μg/ml with the alamar blue assay but only 1 μg/ml MLI with the bioluminescent assay. Also the bioluminescent assay gave early indications of drug-induced bacterial damage as reflected by a reduction in the emission of light before these effects were seen in the viable counts. Amikacin interferes with protein synthesis. In the bioluminescent assay amikacin inhibits the translation of mRNA and thus could result in the production of reduced luciferase as assayed by a reduction in bioluminescence.

Differences in MLI and MRC seen with the bioluminescent and the alamar blue assays respectively presumably reflect differences in the biochemical basis of the assays. The bioluminescent and the alamar blue are indicators of metabolic activity in the host cell. The bioluminescent assay is dependent on $FMNH_2$ but the reduction of the alamar blue occurs via several metabolic intermediates generated by the proliferating cell, including NADPH, FADH and NADH (Mossmann, 1983). Andrews and colleagues (1997) have implicated other factors that are involved in the reduction of alamar blue which may

arise from alterations in intermediary metabolism that are not a reflection of cell lethality. It has been suggested that alamar blue is reduced in the presence of lactate and superoxide.

Although the values obtained with the bioluminescence and alamar blue assays in this study were not compared to standard methods, both methods however have been used for routine MIC determination and compared to standard methods in a number of studies. Using the alamar blue method the drug susceptibility of 50 strains of *M. tuberculosis* was obtained for isoniazid, rifampicin, ethambutol and streptomycin. When MICs were compared with a conventional agar proportion method results for the two methods showed good agreement (Yajko *et al.*, 1995). In addition, the bioluminescence method and the conventional broth macrodilution method were used to evaluate 8 antimicrobial agents against *M. avium*. The MICs determined using the broth macrodilution method were in close correlation with the inhibitory concentrations obtained with the bioluminescence method (Cooksey *et al.*, 1995).

Overall, the alamar blue assay is effective and rapid compared to other assays, and does not require the need for any expensive equipment. The system that is more suitable for high-throughput screening to assay for antimycobacterial activity of compounds against mycobacteria is the bioluminescent assay. Its sensitivity suggests its efficiency for detection of compounds with weaker activity as well as stronger ones. Its rapidity, consistency, ease of use and low cost are important when considering the number of potential compounds to be screened.

6.3. Testing of antimycobacterial drugs against mycobacteria in macrophages.

The aim of this work was to devise a method to test the activity of antimycobacterial agents against recombinant mycobacteria containing the *luxAB* genes, in the murine macrophage cell line J774. The expression of *lux* from bioluminescent mycobacteria treated with antibiotics was measured using a luminoskan luminometer or a charged-coupled device (CCD) camera (Night Owl, Molecular Light Imager, EG and G Wallac Berthold, Wildbad, Germany). Initially, antimycobacterial testing in macrophages was done using only one concentration of drug in order to see if the method developed could work and to obtain technical expertise with the method prior to taking on additional concentrations. After the method had been developed for use in a luminoskan, the

macrophage assay was then used to measure antimycobacterial activity in *M. aurum*:pPA3 infected macrophages using a number of concentrations of amikacin, pyrazinamide or rifampicin. Overall, bioluminescence could be used to evaluate the activity of antimycobacterial agents against recombinant strains of *M. smegmatis* and *M. aurum* containing the *luxAB* genes in macrophages using a luminoskan luminometer.

The J774 macrophages are a continuous reticulosarcoma cell line of murine origin with biological and biochemical characteristics of murine peritoneal macrophages (Snyderman *et al.*, 1977). This cell line permits observation of the effect of antibiotic on intracellular bacteria. Murine peritoneal macrophages have been used to observe the effect of isoniazid and rifampicin on *Mycobacterium microti* infected macrophages (Khor *et al.*, 1986). Thus, this cell line is suitable for use for testing the effect of antibiotics on bioluminescent mycobacteria.

A number of parameters had to be determined in order to devise a method to test antimycobacterial activity against recombinant mycobacteria expressing *lux*. These were the removal of extracellular mycobacteria and lysis of macrophages to release intracellular mycobacteria for subsequent measurement. The monolayer was washed several times and the number of relative light units decreased and eventually stabilised suggesting that the extracellar mycobacteria were effectively being removed. Using this procedure more than 90% of the extracellular mycobacteria were removed from the monolayer and thus demonstrated that this method was optimal. Subsequently, macrophages were lysed using the detergent Saponin followed by sonication. The macrophages were completely lysed when viewed microscopically using both treatments. The effect of centrifugation on the removal of extracellular bacteria and a comparison of disruption methods of J774 macrophages has been reported (Sunderland, *et al.*, 1995). Centrifugation for 2 minutes at 60 g resulted in 2% of extracellular bacteria remaining after washing twice and 0.02% remaining after washing six times. This was achieved since the infection and washing of macrophages was done in suspension and macrophages had not been attached to a surface. In addition, macrophages were completely disrupted by ultrasonication and vortexing with beads and water but treatment with Triton X-100 resulted in incomplete lyses (Sunderland, *et al.*, 1995). The use of detergent and sonication ensures complete lysis of macrophages and disruption of bacterial clumps to allow accurate measurement of bioluminescence.

Although the *lux* system has been used as a reporter to evaluate antimycobacterial activity and gene expression *in vitro*, bacterial *lux* has not been used extensively to study macrophage-microbe interactions. One group has demonstrated the use of bioluminescent bacteria in macrophages to assess its possible application as a reporter of mycobacteriocidal activity. Mycobacteriocidal activity was monitored in *M. smegmatis* (pSMT1) infected J774 macrophages over a period of 72 hours (Snewin *et al.*, 1999). In this work, mycobacteria infected macrophages were assessed over 24 hours. Amikacin was used to remove extracellular mycobacteria and macrophages were lysed using Triton X-100 and subsequently bioluminescence was measured in a luminometer with the addition of aldehyde (Snewin *et al.*, 1999). With *M. smegmatis* infected macrophages measurement of bioluminescence was rapid and simpler than counting colony-forming units (Snewin *et al.*, 1999). This was also the case in this work, bioluminescent readings were obtained within minutes of measurement. With untreated infected macrophages viability of mycobacteria as assessed by colony forming units declined prior to a decrease in bioluminescence and thus suggested that bioluminescence could be a better indicator of metabolic status than viable counting (Snewin *et al.*, 1999). In the results of this thesis, it also appears that bioluminescence is a more sensitive than colony counting since a major effect on bioluminescence was seen with drug treatments that had no effect on colony counting. In addition, after infection of J774s with *M. smegmatis* a decrease in relative light units resulted suggesting some mycobacteriocidal activity was occurring when measured over three days. Amikacin at 200 μg/ml (bacteriocidal) was added to the infected macrophages and incubated for two hours to remove extracellular mycobacteria. Amikacin at 20 μg/ml (bacteriostatic) was added to the macrophages to prevent extracellular growth of mycobacteria released during lysis of macrophages for a period of three days (Snewin *et al.*, 1999). In this work, there was a reduction in bioluminescence from intracellular and extracellar mycobacteria (*M. aurum*) when treated with amikacin at 250 μg/ml and 16 μg/ml suggesting that a reduction in viability of *M. smegmatis* could have been due to the presence of amikacin.

The time limiting factor in these macrophage assays is the removal and lysis of the monolayer to release the internalised bacteria before bioluminescence can be measured in a luminometer. In this work, in order to overcome this factor and consequently reduce time, the wells containing the bioluminescent mycobacteria were measured

directly using a Night Owl CCD camera. Antimycobacterial activity against *M. smegmatis* (pSMT1) and *M. aurum* (pPA3) was demonstrated by reduced bioluminescence after addition of rifampicin or streptomycin. This method could detect antimycobacterial activity against mycobacteria but with much reduced time and handling than previously required for assessment of bioluminescence.

Several groups have used a CCD camera for the measurement of light in their studies. Using a CCD luminescence imager (Photonic Science) bioluminescent *Bordetella bronchiseptica* could be detected (Forde *et al.*, 1998). The complete *lux* operon from *P. luminescens* was used to construct mini-Tn5 promoter probe vectors that were used to measure intracellular survival and persistence of *Bordetella* within murine macrophages. After transformation of *Bordetella* with pUTmini-Tn5km*lux*, *lux* positive phenotypes were detected using a CCD luminescence imager. The CCD imager was able to detect a number of bioluminescent bacteria that varied in their expression of bioluminescence. Murine macrophages were then infected with one strain of *Bordetella* found to be highly bioluminescent and a luminometer was used to measure the light produced from the recombinant *Bordetella* infected macrophages. Light could be detected from the infected macrophages over a period of time indicating that *B. bronchiseptica* could survive and persist within the macrophage and suggested that bioluminescence was effective as a reporter system. Although, the light output was found to be higher in the absence of macrophage since only 1 to 2% of bacteria were internalised, intracellular bacteria could still be detected using a luminometer.

The Night Owl CCD camera has also been used to detect bioluminescent *Brevundimonas diminuta* with *luxCDABE* or *luxAB* from *P. luminescens* integrated into the chromosome in order to test the effectiveness of sterilisation-grade filter membranes (Griffiths *et al.*, 2000). After the membrane was incubated for 24 hours, detection sensitivity using the Night Owl CCD camera was 77% and 84% respectively, while at 48 hours this increased to 100% for each strain (Griffiths *et al.*, 2000). A scan time of 60 seconds gave the highest detection sensitivity and this scan time was also used to measure antimycobacterial activity against mycobacteria in this work. The advantage of using the Night Owl CCD camera was the low level background produced compared to other CCD cameras, such as the Nucleovision CCD camera that produced greater background and thus reduced the signal obtained by the bioluminescent colonies

(Griffiths *et al.*, 2000). The Night Owl CCD camera was found to be very sensitive in detecting bioluminescent bacteria; $1x10^4$ bioluminescent bacteria could be detected. Also data were presented very quickly and the levels of background detected from uninfected macrophages were very low.

The rapidity in obtaining results when using a CCD camera has been demonstrated in the following study (Masuko *et al.*, 1991a). Bioluminescent *Photobacterium* were suspended in medium and placed on a membrane filter and bioluminescence was measured using a C2400-20H Hamamatsu ARGUS-100 camera. Using this method more than 70% of bright spots could be detected after an hour including the detection of a single bacterium. Incubation of the membrane confirmed that the bioluminescence spots correlated with the number of bacterial colonies (Masuko *et al.*, 1991a). The time taken for one sample to be read required 50 minutes image integration and this was reduced to 10 minutes image integration in a further study (Masuko *et al.*, 1991a; Masuko *et al.*, 1991b). Thus, this method is both rapid and sensitive. However, in this work we have shown that further improvement in rapidity was possible and that samples could be read in one minute and images processed almost immediately.

When using the luminoskan luminometer, decanal has to be dispensed into the wells of the microtitre plate containing the lysed macrophages and bacteria and this resulted in the contact of bacteria to decanal throughout the experiment and also increased the volume in the wells thus, reducing the opportunity for further measurement at a later time. The CCD camera could be used to observe antimycobacterial activity against mycobacteria within macrophages over a longer period of time. The aldehyde does not have to be added into the medium containing the intracellular mycobacteria for detection of bioluminescence using the CCD camera and therefore this method could be used for the continuous assessment of mycobacteria infected macrophages. The decanal is added to the lid of the 24-well plate and vaporises, subsequently contact with intracellular mycobacteria containing the *lux* genes results in light production. The advantage of this method is that the decanal does not cause toxicity since it can be removed and replaced with a fresh lid and bioluminescence measured again at a later time. In addition, both the CCD camera and the luminoskan luminometer measure bioluminescence of the total amount of bacterial mass within a population of macrophages.

Detection of bioluminescent viruses expressing firefly luciferase within a single HeLa cell over a period of time has been demonstrated with a CCD camera and a microscope attachment (White *et al.*, 1995). This study demonstrated the possibility of monitoring the activation of luciferase reporter enzyme expression at different time intervals following activation of gene expression in a single HeLa cell containing bioluminescent bacteria. Bioluminescence was measured in single cells over a period of 27 hours and was found to increase over this time period demonstrating that this method could be used as a non-destructive, real-time measurement of gene expression within a single cell (White *et al.*, 1995).

In general, the use of *lux* as a reporter system has several advantages. First the bioluminescent reaction is very efficient and also the natural bioluminescent background is negligible from bacteria and macrophages and thus enables its use in the study of macrophage-microbe interactions. The luminoskan luminometer is an efficient way of screening for antimycobacterial activity against mycobacteria infected macrophages for use in high-throughput screening since it is automated and easy to use. However, the high sensitivity of low light level imaging cameras, the relative ease of use and the ability to measure continuous expression in a population of cells or within single cells without disruption of macrophages, makes the CCD camera more attractive for use than the luminometer in some situations. The major disadvantage of the CCD camera is that they are very expensive. Overall, it has been shown that the method developed could be used as a model to test the activity of antimycobacterial agents against bioluminescent mycobacteria within the murine macrophage cell line J774. Given that bioluminescent *M. smegmatis* and *M. aurum* were used in these studies, further testing could to be done using bioluminescent *M. tuberculosis*. To date, no studies have demonstrated use of the complete bacterial *lux* operon in *M. tuberculosis* but in this work bioluminescent *M. tuberculosis* containing the entire *lux* operon from *P. luminescens* was produced but was found to be unstable. Further work could be done to try and generate stable *Mycobacterium tuberculosis* transformants and furthermore, testing of novel antimycobacterial agents against murine macrophages could be preliminary before testing in an animal model.

6.4. Acid stress in mycobacteria.

The aims of the work described in chapter 4 and chapter 5 were:

- To construct a promoter probe vector based on the *lux* reporter system.
- To use this vector to assay *in vitro* regulation of expression of two genes identified from *Mycobacterium tuberculosis*, Rv2531c and *phoPR*, which are thought to be inducible by low pH and/or involved in the acid tolerance response
- To confirm that *M. smegmatis* did, and to determine whether BCG, possessed an acid tolerance response.
- To construct a knockout mutation of the Rv2531c gene (putative decarboxylase) in *Mycobacterium smegmatis*, in order to see if this gene was involved in the acid tolerance response (ATR).

6.4.1. Identification of genes regulated by pH and/or involved in the acid tolerance response.

Before the aims described above were done an attempt to identify acid tolerance response genes of *M. tuberculosis in silico* was done. The promoter of these genes would then be placed upstream of a *lux* based reporter plasmid. Several genes from *E. coli* and *S. typhimurium* had been identified with these properties and were used to find sequences with similarity in *M. tuberculosis*. Two genes with sequence similarity were identified in *M. tuberculosis*, a two-component regulatory system, *phoPR* and a putative decarboxylase gene, Rv2531c. *phoPR* was chosen since an acid-inducible fragment (ORF-1) (Gordon, 1995) identified from *M. smegmatis* was identified as being for *phoR* and consequently the industrial collaborators requested that the study of this two-component system be undertaken. Furthermore, *phoPQ* from *S. typhimurium* (analogous to *phoPR*) is a regulator of acid tolerance. The *M. tuberculosis* gene sequence homologous to *phoPQ* from *S. typhimurium* was identified on cosmid MTCY369.03. The product of this gene was annotated as PhoR.

The two-component regulatory system PhoPR in *M. tuberculosis* is similar to that of PhoPQ of *S. typhimurium*. In *M. tuberculosis* and *S. typhimurium*, PhoP is a transcriptional regulatory protein (activator) and PhoR and PhoQ is a sensor-kinase

protein respectively. The gene upstream of *phoPR* in *M. tuberculosis* codes for PE-PGRS; part of the PE protein family and the gene downstream of *phoPR* in *M. tuberculosis* is *aldA*, a probable aldehyde dehydrogenase.

Several inducible amino acid decarboxylases play a role in the maintenance of intracellular pH in *E. coli* (Gale, 1946). In particular, the lysine decarboxylase gene system of *E. coli* has been widely described (Tabor *et al.*, 1980; Auger *et al.*, 1989; Watson *et al.*, 1992; Meng and Bennett, 1992a). The lysine, arginine and ornithine gene sequences from *E. coli* were matched to the *M. tuberculosis* gene sequence and all three genes had sequence similarity in *M. tuberculosis* to one particular gene sequence, identified on cosmid MTCY159.25 (Rv2531c). The product of this gene was unknown at the start of this work.

At the end of the project, the two genes ORF-1 and Rv2531c were matched again to the *M. tuberculosis* genome sequence (Appendix 1). When a new search with ORF-1 from *M. smegmatis* (Chapter 4., Figure 4.1.,) was done to the *M. tuberculosis* H37Rv genome sequence (http://www.sanger.ac.uk), new sequences with homology to ORF-1 were found. Originally with ORF-1, two sequences were found, on cosmids MTCY10G2.17 and MTCY369.03. The new blast search revealed a third gene on cosmid, MTV025.112c that also was identified as a two-component regulatory system and annotated as a hypothetical protein, Rv3764c. MTCY10G2.17 also codes for a hypothetical protein, Rv1032c.

When the searches for the inducible amino acid decarboxylases in the *M. tuberculosis* genome sequence were repeated, all three decarboxylases were again identified with sequence similarity to MTCY159.25. The gene was annotated Rv2531c or *adi*. No other genes from *M. tuberculosis* were found to have sequence similarity to the decarboxylases of *E. coli*.

The genes *cadC* and *cadB* from *E. coli* were also matched to the *M. tuberculosis* gene sequence because *cadB* forms an operon with *cadA* and *cad*C is involved in the activation of P*cad* that subsequently leads to the transcription of this operon. The gene *cad*C from *E. coli* that codes for a transcriptional activator of P*cad* was found to have some sequence similarity in the *M. tuberculosis* genome sequence. These included a

response regulator identified as RegX3 (MTCY20G9.17) and a putative transcriptional activator, MtrA (MTCY20B11.21c). The *cadB* gene from *E. coli* coding for a transport protein for the substrate (lysine)/end product was also found to have some sequence similarities in the *M. tuberculosis* genome sequence. These included a hypothetical protein, Rv1999c, a transport protein identified on cosmid MTCY39.19 and also to a D-serine/D-alanine/glycine transporter, annotated as *cycA* on cosmid MTCI125.26c.

As mentioned earlier, in *E. coli*, *cadA* and *cadB* form an operon and situated upstream of these two genes is the promoter P*cad* and upstream of P*cad* is *cadC* (Watson *et al.*, 1992; Meng and Bennett, 1992a). A similar arrangement of decarboxylase and transport genes has been found for the inducible ornithine decarboxylase. The two genes, *speF* encoding the biodegradative ornithine decarboxylase and *potE* encoding a membrane-bound putrescine-ornithine antiporter are part of an operon that is induced at a low pH (Kashiwagi *et al.*, 1991; 1992). From the *M. tuberculosis* sequence there was no suggestion that the putative decarboxylase Rv2531c formed an operon. Both genes upstream and downstream of the *adi* gene in *M. tuberculosis* have no known function. The gene upstream of *adi* is a protein of unknown function and the gene downstream of *adi* is a conserved hypothetical protein, also of unknown function. Genes either side of these genes code for nitrogen-utilisation substance protein B (*nusB*), elongation factor P (*efp*) and restriction system protein (*mrr*). Thus, this arrangement in *M. tuberculosis* does not compare with the *cadBA* or *speF/potE* operon from *E. coli*. The inducible arginine decarboxylase encoding *adi* from *E. coli* does not appear to be part of an operon involving upstream regions (Stim and Bennett, 1993). A downstream fragment however, exhibits pH induction of β-galactosidase when cloned into a fusion vector thus it is possible that this downstream region may contain a transport-related gene or a gene that has a regulatory role (Stim and Bennett, 1993).

At the end of project the blast searches of the genes from *E. coli* and *S. typhimurium* against the *M. tuberculosis* genome sequence were repeated (Appendix 1). Previously, three of the genes were found not to have any sequence similarity to the *M. tuberculosis* genome sequence. These included *icd* (isocitrate dehydrogenase), *purB* (adenylosuccinate lyase) and *osmY* (unknown function). However, after matching these gene sequences to the *M. tuberculosis* genome sequence at the end of project, sequence similarities were found for these three genes. The gene *icd* from *E. coli* was found to

have 35% identity to the gene *leuB* (MTV012.09) coding for 3-isopropylmalate dehydrogenase (isocitrate dehydrogenase) from *M. tuberculosis*. The gene *purB* from *E. coli* was found to have 28% identity to the gene *purB* (MTCY369.21b) coding for adenylosuccinate lyase from *M. tuberculosis* and the gene *osmY* from *E. coli* was found to have 31% identity to a gene coding for a hypothetical protein Rv1008 from *M. tuberculosis*.

6.4.2. *Mycobacterium smegmatis* and *M. bovis* BCG and the acid tolerance response.

A model to test for an acid tolerance response in mycobacteria has come from extensive work done by Foster and others (Foster and Hall, 1990; Foster, 1991; Foster, 1995; Bearso, 1997). The model proposes that bacteria are able to survive exposure to an extreme acidic pH following adaptation at a relatively mild acidic pH. This is a two-stage process involving the synthesis of emergency pH homeostasis systems that alkalinise the cytoplasm during periods of mild acid stress, also known as pre-acid shock. The post-acid shock is the second stage that occurs once external pH falls to or below 4.5 and increases the organisms acid tolerance to further challenge at an extreme acid external pH of around 3.3. Acid shock proteins are produced during the post-acid shock stage and at pH 3.3, to prevent and/or repair damage caused by acid shock (Foster and Hall, 1990; Foster, 1995; Bearso, 1997). Work done on the response of *M. smegmatis* to acid stress from O'Brien and others (1996) has been used as the basis to test the hypothesis that two genes, Rv2531c and *phoPR* identified from *M. tuberculosis*, could be involved in acid tolerance in mycobacteria by testing for increased expression of the *lux* reporter system downstream of the promoter for these two genes and by constructing a knockout mutation of the Rv2531c gene. Time constraints prevented construction of the *pho* knockout mutation.

However before working with the transformants it was decided that the data with *M. smegmatis* should be confirmed with *M. smegmatis* (pJS12) (P*pd*), *M. smegmatis* (pJS13) (*PphoPR*) and BCG. Thus the adaptive and lethal pH had to be determined as done by Foster and Hall (1990). An acid tolerance response with BCG had previously not been determined. This experiment with the two strains of *M. smegmatis* was done to confirm previously identified adaptive and lethal pHs as done by O'Brien and others (1996) and to show that the plasmids did not have an effect on the acid tolerance

response of *M. smegmatis.* In addition, these adaptive and lethal pHs identified would be used to determine whether the promoters for the two genes Rv2531c and *phoPR* were acid-inducible. The experimental procedures for BCG were based on modifications of *M. smegmatis* procedures. *Mycobacterium smegmatis* (pJS12), *M. smegmatis* (pJS13) and BCG were grown at pH ranging from pH 7.6 to pH 2.5 with the medium acidified with hydrochloric acid (HCl) or phosphoric acid (H_3PO_4). The adaptive pH was defined as the lowest external pH at which bacteria continued to replicate and the lethal pH was defined as the lowest external pH that caused significant decrease in viability (Foster and Hall, 1990).

The selection of the two acids, hydrochloric acid and phosphoric acid was based on the idea that different acids could result in different degrees of acid tolerance. When three organic acids were compared, acetic, citric and malic acid, adapted *E. coli* O157:H7 showed differences in survival to these acids demonstrating that acid tolerance was affected by the type of acidulant (Deng *et al.*, 1999). It could be argued whether the results obtained here were dependent on pH or whether they were simply attributable to the anion. It is possible for the inorganic components of the medium to interact with adverse pH to affect bacterial growth and survival, both positively and negatively. Rowbury and others (1992) demonstrated that phosphate ions prevented habituation at low external pH in *E. coli.* They suggested that habituation involved hydrogen ions crossing the outer membrane possibly via the PhoE pore, a process inhibited by phosphate and other anions. Rowbury and others suggested that in Foster and Hall's (1990) experiments, the medium used for acid tolerance response (ATR) induction contained substantial amounts of phosphate that would have abolished habituation in *E. coli.* Thus findings suggest that habituation in *E. coli* and the ATR of *S. typhimurium* have major differences. An acid tolerance response was observed in mycobacteria using phosphoric acid and therefore the concentration of phosphate in the medium was not sufficient to have such an effect. Also, if an acid tolerance response had only been observed in the presence of phosphoric acid and not with hydrochloric acid, then it may have been possible that phosphate was affecting acid tolerance in mycobacteria. An acid tolerance response however, was detected using both acids. However, in the environment it is possible that other ions may modify the stress imposed by the actual pH. Future experimentation could be done to measure the sensitivity of mycobacteria to inorganic acid by exposing cultures to broth acidified with hydrochloric acid and in the

presence of different concentrations of phosphate and observing for survival after acid challenge.

It is important to consider not only the effects of the anion on acid tolerance but also the possible osmotic effects or modifications in labile medium components, for example catalase, resulting from very low pH. Osmolarity can effect gene expression. Numerous studies previously done to measure the acid tolerance response in several organisms have not described the effects of osmolarity. One way to measure the effect of osmolarity or pH could be to measure changes in DNA supercoiling. Cultures grown in high osmolarity leads to an increase in negative DNA supercoiling whilst cultures grown at acidic pH result in decreased negative DNA supercoiling (Karem and Foster, 1993). A modification in one medium component, catalase, resulting from very low pH has also been considered. Catalase converts hydrogen peroxide into water and oxygen. It is possible that acid tolerance could occur due to adaptation to hydrogen peroxide as a result of altered catalase activity. Goodson and Rowbury (1989) however, exposed *E. coli* to medium acidified with inorganic acid in the presence and absence of catalase and demonstrated that acid resistance of *E. coli* was not due to greater resistance to hydrogen peroxide that was present in the medium.

The adaptive pH for *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) using HCl was identified as between pH 4.5 and pH 4.0 and the lethal pH for both strains was identified as pH 2.5. With BCG the identified pHs were much higher with HCl as the acidulant, with the adaptive pH identified as between pH 6.6 and pH 5.0 and the lethal pH was identified as pH 3.0. The adaptive pH for *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) using H_3PO_4 was identified as between pH 5.0 and pH 4.5 and the lethal pH for both strains was identified as pH 2.5. However with BCG, using H_3PO_4 as the acidulant, the adaptive pH was between pH 4.5 and pH 4.0 and the lethal pH was identified as pH 3.0.

O'Brien and others (1996) reported the adaptive pH for *M. smegmatis* was pH 5.0 with both HCl and H_3PO_4 as the acidulants. The lethal pH was 3.0 and 3.5 when using HCl and H_3PO_4 as the acidulants respectively (O'Brien *et al.*, 1996). Although some variations in the identified adaptive and lethal pHs are evident in *M. smegmatis* (pJS12), *M. smegmatis* (pJS13) and *M. smegmatis*, these differences may be due to

experimental variation or technical difference. In our work, for example, cells were centrifuged and added to the acidified medium whilst O'Brien and others added acid directly into the medium for acidification. Experimental variation using the same organism has been observed elsewhere. In one study, pH 5.0 and pH 3.0 were chosen as the adaptive and lethal pHs respectively for *Listeria monocytogenes*, whilst in another study pH 5.5 and pH 3.5 were chosen (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996).

The adaptive pH identified for *M. smegmatis* (pJS12), *M. smegmatis* (pJS13) and BCG were found to be different to the adaptive pH of other bacteria. For example, the adaptive pH for *Salmonella typhimurium* was pH 5.8, pH 5.0 for *E. coli* and *Aeromonas hydrophila*, pH 5.3 for *Enterococcus hirae* and pH 4.5 for *Propionibacterium freudenreichii* with HCl acid as the acidulant (Goodson and Rowbury, 1989; Foster and Hall, 1990; Karem *et al.*, 1994; Jan *et al.*, 2000). The lethal pH for other bacteria also was different, with the lethal pH as high as 4.4 for *Vibrio parahaemolyticus*, pH 3.3 for *S. typhimurium*, pH 3.0 for *Escherichia coli* and as low as pH 2.5 for *Streptococcus mutans* with HCl acid as the acidulant (Goodson and Rowbury, 1989; Foster and Hall, 1990; Belli and Marquis, 1991; Wong *et al*, 1998). These experiments demonstrate the wide variation in the adaptive and lethal pHs among these different bacteria. This could be due to different environmental conditions encountered by these bacteria. Although as discussed, experimental variation may also contribute.

It has been reported that fast growing mycobacteria have a wider pH range of growth compared with slow growing mycobacteria (Portaels and Pattyn, 1982). Surprisingly however, BCG sensitivity was similar to *M. smegmatis.* This range of pH tolerance was wider than previously reported with BCG and other slow-growing mycobacteria (Chapman and Bernard, 1962). For example, with BCG optimal growth was reported as between pH 5.4 and 6.5 and with partial growth at pH 5.0. No growth occurred below pH 5.0 (Portaels and Pattyn, 1982). In a recent study, BCG was shown to grow at a pH value of 5.0 to 7.0, but with no growth at pH 4.5 (Rao *et al.*, 2001). With *M. tuberculosis*, maximal growth was identified as between pH 6.2 and 7.3, and with very low levels at pH 5.0. No growth was seen at pH 4.0 to pH 3.0 (Chapman and Bernard, 1962). These studies suggest that BCG and other slow growers prefer to grow in slightly acidic conditions compared with the fast growers (Portaels and Pattyn, 1982). In our work, results demonstrate that overall fast and slow growers grow at acidic pHs.

With *M. smegmatis* optimum growth was reported to be between pH 7.4 and pH 5.0 and partial growth at pH 4.6 (Portaels and Pattyn, 1982). In our work, with the recombinant mycobacteria optimum growth was observed between pH 7.6 and pH 4.0 with HCl and pH 7.6 and pH 4.5 with H_3PO_4 as the acidulant. *M. smegmatis* has been reported to grow at a wider pH range between pH 3.5 to pH 9.5, although cultures when observed at pH 3.0 were not viable (Chapman and Bernard, 1962). In comparison it was found that in our work, growth occurred at pH 4.0 to pH 7.6, although viable bacteria were observed at pH 2.5 for *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13).

pH readings taken at 4 weeks by Chapman and Bernard (1962) demonstrated an elevated pH due to products of metabolism from pH 4 to pH 5.4 and from pH 5.0 to pH 6.0. During this study, pH readings remained the same ± 0.5 units after 6 hours.

A further report indicated intracellular pH homeostasis in *M. smegmatis was* critical for the survival of these bacteria at acidic pH values (Rao *et al.*, 2000). External pH was adjusted using HCl in the range pH 7.0 to pH 4.5. Maximum growth rate for *M. smegmatis* was seen at pH 7.0 and growth rate declined as pH decreased (Rao *et al.*, 2000). *M. smegmatis* were reported to maintain an internal pH in the range 6.1-7.2 when presented with an external pH range of 7.0-4.0 (Rao *et al.*, 2000). In our work, when the pH of the medium was dropped below 4.0 using HCl, growth rate reduced and cells remained viable below this pH. This is supported by the findings of Rao and others who showed that at an external pH of 4.0, *M. smegmatis* were unable to grow although they that could maintain an internal pH of greater than 6.0 (Rao *et al.*, 2000).

Mycobacterium smegmatis (pJS12), *M. smegmatis* (pJS13), BCG (pJS12) and BCG (pJS13) possess an acid tolerance response. Both recombinant *M. smegmatis* incubated at the adaptive pH using HCl or H_3PO_4 as the acidulant, demonstrated enhanced survival over six hours when challenged at the lethal pH compared with unadapted cultures. Thus, protection occurs with two types of acidulant. O'Brien and others (1996) have demonstrated an acid tolerance response in *M. smegmatis* using HCl and H_3PO_4 as the acidulants. Cultures exposed to a lethal pH of 3.0 following adaptation at pH 5.0 with HCl demonstrated an ATR in *M. smegmatis.* With H_3PO_4, cultures adapted at pH 5.0 survived significantly better at a lethal pH of 3.0 than unadapted cultures over four hours. Thus, the recombinant mycobacteria and *M. smegmatis* both possess an ATR.

Generally adapted cultures of *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) were up to 3-4 times more resistant to acid stress after exposure to the lethal pH compared with unadapted cultures. The magnitude of protection for adapted *M. smegmatis* was 2-3 fold greater than unadapted cultures (O'Brien *et al.*, 1996).

Adapted cultures of recombinant *M. smegmatis* were compared at pH 4.5 using HCl or H_3PO_4 as the acidulants, to determine whether variations in acid tolerance were dependent on the acidulant. A significant ($P< 0.05$) difference was seen between HCl and H_3PO_4 with recombinant *M. smegmatis* at pH 4.5. O'Brien and others also tested acid tolerance in *M. smegmatis* using HCl and H_3PO_4 and their results appear to show a difference in the two acidulants. Thus, while acid tolerance is seen in *M. smegmatis,* it is dependent on the type of acidulant.

BCG (pJS12) and BCG (pJS13) possess an acid tolerance response with HCl as the acidulant. BCG (pJS12) and BCG (pJS13) survived significantly better at pH 3.0 following adaptation at pH 6.6 compared with unadapted cultures grown at pH 7.6. Using H_3PO_4 as the acidulant no significant differences were seen when cultures of BCG (pJS12) and BCG (pJS13) were adapted at pH 5.5, pH 5.0, pH 4.5, or pH 4.0 followed by exposure to pH 3.0 compared with unadapted cultures. Thus, there was no statistically significant acid tolerance response using H_3PO_4 as the acidulant. However, within the data there is the suggestion that an acid tolerance response may be possible with H_3PO_4. Although, the data do not reach statistical significance, survival at pH 3.0 seems to be better after a period of moderate pH. Further replicates would be required to confirm this suggestion. However, the conclusion at the moment must be that a H_3PO_4 induced acid tolerance response does not occur in BCG.

Adapted recombinant BCG were only two to three times more resistant to acid stress than the control and this was comparable with adapted and unadapted *M. smegmatis.* Although *M. smegmatis* (pJS12), *M. smegmatis* (pJS13), BCG (pJS12) and BCG (pJS13) did exhibit an acid tolerance response, this level of protection was considerably lower compared with other adapted bacteria. For example, over 1000-fold increase was seen with *S. typhimurium* adapted at pH 5.8 with HCl and acid challenged at pH 3.3 compared with unadapted cultures (Foster and Hall, 1991). *Lactococcus lactis* showed 100% resistance to acid challenge at pH 4.0 following adaptation at pH 5.0 for one hour

compared with less than 2% survival for unadapted cultures (O'Sullivan and Condon, 1997). Seventy eight percent of *P. freudenreichii* survived acid challenge at pH 2.0 after sixty minutes following adaptation at pH 4.5 compared to approximately 0.01% survival for unadapted cells (Jan *et al.*, 2000).

With H_3P0_4 as the acidulant, *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) both had enhanced survival at pH 2.5 when exposed to the adaptive pH of 4.5 and 5.0. However, exposure to pH 4.5 appears to produce even better acid tolerance than pH 5.0. This phenomenon of enhanced acid tolerance due to a decrease in pH has also been observed in other studies. With *P. freudenreichii*, an acid tolerance response with adaptation at pH 5.0 resulted in sixty nine percent survival whilst at pH 4.5, seventy eight percent survival occurred (Jan *et al.*, 2000). Furthermore, *L. lactis* had increased ability to survive acid challenge as the external pH was decreased from pH 7.0 to pH 5.0 (O'Sullivan *et al.*, 1997).

Originally, when testing for an acid tolerance response in BCG, cultures were exposed to a lethal pH of 2.5 with HCl following adaptation. No acid tolerance was exhibited with BCG but cultures adapted at pH 5.0 or pH 4.0 showed extensive clumping of the cells at pH 2.5 after incubation for twenty four hours whilst unadapted cultures remained clump-free. This suggested under these conditions a change to the membrane surface of the cell in BCG had occurred indicating an acid adaptive response. This extensive clumping was not seen with adapted cultures of *M. smegmatis* at pH 2.5. Growth at acidic pH has been shown to cause changes in lipid composition of *Clostridium acetobutylicum* and *Staphylococcus aureus* (Booth, 1999). Nevertheless, the surface properties of BCG and *M. smegmatis* have been reported to be identical in terms of electrokinetic properties, irrespective of the growth medium, age of the cells and various chemical treatments (Hardham and James, 1981). The negative charge on the surface of BCG increased rapidly from pH 2.0 to pH 4.0 and more steadily to pH 9.0 but no disorganisation of the cell surface was observed when BCG was exposed to pH 2.0 to pH 9.0 (Hardham and James, 1981). However, the forces of attraction between positively charged groups on one cell and negatively charged groups on another cell were negligible since no cationic surface groups were detected (Hardham and James, 1981). It has been suggested that aggregation displayed by BCG is possibly due to the secretion of a 'slime-like' material which covers groups of cells and binds them together

(Hardham and James, 1980; Hardham and James, 1981). This covering material may well prevent extensive damage to the cell from exposure to acid.

Overall, this work has demonstrated the presence of an acid tolerance response in *M. smegmatis* and in BCG. This is the first report of an acid tolerance response in BCG with hydrochloric acid as the acidulant. BCG and *M. smegmatis* can survive at an extreme pH only if they are initially adapted to a mild pH.

6.4.3. Is P*pd* acid-inducible in *M. smegmatis*?

Recent studies have reported the identification of several genes, particularly from *S. typhimurium* and *E. coli*, whose expression is induced or enhanced at acid pH (Slonczewski and Foster, 1996). Based on these findings the aim of this work was to test a hypothesis that certain genes from *M. tuberculosis* were also induced under external acidic conditions. The promoters of two of these genes Rv2531c (coding for a putative decarboxylase) and *phoPR* (coding for a two-component regulator) were studied in order to determine whether they were acid-inducible and if they were involved in the acid tolerance response. P*pd* has only been tested in *M. smegmatis* and not in BCG due to lack of time.

Many studies have employed the use of the *lac* fusion techniques to observe the regulation of gene induction by internal and external pH (Auger *et al.*, 1989; Park *et al.*, 1996). This technique has been used to identify decarboxylase genes in *E. coli* and the promoter responsible for pH-regulated expression (Meng and Bennett 1992; Watson *et al.*, 1992). The promoter probe vector constructed for use in mycobacteria in this work was based on *lux*. The promoter for the genes Rv2531c and *phoPR* were placed upstream of *luxAB*. The *lux* reporter system was used to determine if these promoters could induce the expression of *lux* under acidic conditions. This system was used over the *lac* system for a number of reasons. Bioluminescence could be measured directly from the intact cells and this system did not require the disruption of mycobacterial cells in order to assess the expression of *lux*, which therefore made this method more practical. This method is specific, since bioluminescence would only be produced from the recombinant mycobacteria containing *lux* genes and also results could be obtained within minutes. A previous study demonstrated the effectiveness of luciferase as a reporter of mycobacterial promoter activity (Gordon, 1994). The *M. smegmatis*

acetamidase promoter was placed upstream of *lux* and upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and a comparison of the two systems were made (Parish, 1993; Gordon *et al.*, 1994). The promoter was induced 1000-fold as seen with *lux* compared to only 10-15 fold increase with CAT demonstrating that the *lux* reporter system was more sensitive for the identification of mycobacterial promoters (Parish, 1993; Gordon *et al*, 1994). Also, the *lux* system is more rapid than the CAT assay and is also non-radioactive (Promega, 1991).

Extreme external acidification can overload the pH homeostasis and substantially depress internal pH. The decarboxylation of amino acids, for example, that of lysine to cadaverine via the *cad* system in *E. coli* (Meng and Bennett, 1992; Watson et al., 1993), or of arginine to agmatine via arginine decarboxylase (Stim and Bennett, 1993), or of ornithine to putrescine via ornithine decarboxylase leads to an increase in external pH, and are responses induced by low pH. Several other decarboxylases show a similar pattern of regulation.

To test whether the Rv2531c promoter, P*pd* was induced under acidic external adaptive pH, *M. smegmatis* (pJS12) was grown at pH 7.6 and subsequently exposed to the adaptive pH and observed for increased expression of *luxAB*. When *M. smegmatis* (pJS12) was exposed to a pH of 4.5 using HCl or H_3P0_4 as the acidulants, significantly increased levels of bioluminescence were seen using both acids compared with unadapted cultures. Significant ($P< 0.005$ with HCl and $P< 0.001$ with H_3P0_4) levels of expression of *lux* were detected only after twenty four hours of exposure. Approximately 200-fold increase in light production was seen at pH 4.5 after exposure for forty eight hours after cultures were dropped from pH 7.6 with both acids, indicating that P*pd* was induced under acidic external conditions. Although low levels of bioluminescence were detected during the first eight hours of exposure from *M. smegmatis* (pJS12), these levels were not significantly ($P> 0.05$) different between cultures adapted at pH 4.5 and those unadapted at pH 7.6.

Since the expression studies indicated that P*pd* was not expressed during the same time at which an acid tolerance response was exhibited with *M. smegmatis* (pJS12), it is possible that increased expression of Rv2531c is not necessarily required in the induction of the acid tolerance response of *M. smegmatis.* However, this does not mean

that Rv2531c is not involved in the acid tolerance response. These data indicate that Rv2531c is induced under acidic external conditions and therefore Rv2531c is an acid response gene. Conversely low-level expression without induction may also be enough for involvement in the acid tolerance response. Genes that sense and/or respond to acidification are not always necessarily involved in the acid tolerance response. There are acid regulated genes for example; *aniG* and its regulator *earA*, that when mutated the host bacterium (*S. typhimurium*) still exhibits a significant acid tolerance response (Foster and Hall, 1990).

There are several inducible decarboxylases, some of which include *adi* encoding arginine decarboxylase, *cadA* encoding lysine decarboxylase, *gad* encoding glutamate decarboxylase, and *dcor* encoding ornithine decarboxylase from *E. coli*. Evidence is available demonstrating that these amino acid decarboxylases are induced by low pH. This phenomenon has been known for several years and was initially reported by Gale (1946) who showed that when *E. coli* were grown at pH ranging from 8.5 to 5.0, the activity of several amino acid decarboxylases increased with decreasing pH with maximum activity after sixteen hours (Gale, 1946). The expression of amino acid decarboxylases is affected by pH, substrate and availability of oxygen. Maximum expression is observed when cells are grown at low pH, with excess substrate and grown anaerobically (Auger *et al.*, 1989).

The expression of an amino acid decarboxylase promoter in *E. coli* upon changes in external pH has been studied using a series of vectors with *lac* (Tolentino *et al.*, 1992). When the pH of the cultures was lowered from pH 7.0 to pH 6.5 to pH 5.5, the levels of β-galactosidase increased with decreasing pH over four hours. The optimum level of expression occurred at pH 5.5 with approximately 60-fold increase in induction. In *M. smegmatis,* P*pd* did not behave exactly as reported with *E. coli*, in that, no significant increase in the expression of *lux* occurred over four hours. The *cad*A promoter in *E. coli* was also shown to respond to increasing pH by decreasing the level of expression (Tolentino *et al.*, 1992). Furthermore, lysine decarboxylase from *S. typhimurium* was induced in the presence of low pH (Park *et al.*, 1996). Cultures grown at pH 7.7, and adapted at pH 4.4 were able to effectively undergo acid tolerance at pH 3.0 in the presence of lysine. Adapted cells survived almost completely compared with unadapted cells following acid challenge for ninety minutes (Park *et al.*, 1996). However, in *M.*

smegmatis increased expression of *lux* under the control of the Rv2531c promoter did not coincide with acid tolerance, suggesting that P*pd* may not be crucial in the induction of the acid tolerance response in contrast to *S. typhimurium*.

No induction of expression of *lux* downstream of P*pd* was seen during the first eight hours of exposure at pH 4.5 with either hydrochloric or phosphoric acid. Thus, either Rv2531c is not activated in *M. smegmatis* by low pH during this time or that the internal pH is maintained during this time and therefore there is no expression of *lux*. The induction of P*pd* expression after twenty-four hours could occur in response to a decrease in internal pH demonstrating that P*pd* responds to acid. Amino acid decarboxylases are only activated after the internal pH drops beyond a certain pH (Gale, 1946). The lower the pH during growth the greater the formation of the amino acid decarboxylase. Lysine, arginine and ornithine decarboxylases however, are not formed when growth takes place at an alkaline pH (Gale, 1946). The pH optimum of one such enzyme, lysine decarboxylase was determined experimentally to be 5.7 (Sabo *et al.*, 1974). In V. *cholerae* however, *cad*A transcription was greatest only when cells were shifted from neutral (pH 7.0) to an acidic pH of 4.5 following adaptation at pH 5.7 for one hour using hydrochloric acid. No transcription was seen at pH 7.0 or pH 5.7 demonstrating that *cad*A was induced under specific conditions of low-pH (Merrell and Camilli, 1999; 2000). Gale (1946) demonstrated that the amino acid decarboxylases were active over a narrow range of pH with optima in all cases lying between pH 2.5 and 6.0. For example, pH optimum for lysine decarboxylase was 4.5-5.0 and pH 4.0-4.8 for arginine decarboxylase. Low specific activity of amino acid decarboxylases at neutral pH could be due to pH-dependent properties, concentration or post-translational processing (Merrell and Camilli, 2000). This suggests that *M. smegmatis* are able to maintain a high internal pH during external acidic conditions and thus there is no observable expression of *lux* downstream of P*pd*.

Why is P*pd* not expressed at the same time as the acid tolerance response with phosphoric acid as the acidulant? It is possible that phosphate prevents proton entry into the cell by blocking proton pores and thus the cell is able to maintain a high internal pH. Rowbury and Goodson (1992) have shown that phosphate prevents entry of protons into the cell by competing with proton ions for the PhoE pore. Phosphate has shown to inhibit the induction of one particular amino acid decarboxylase, *cad*A encoding lysine

decarboxylase (Rowbury and Goodson, 1993). Also the amino acid decarboxylases are not activated during high internal pH (Gale 1946). *M. smegmatis* can maintain an internal pH of greater than 6.0 at an external pH of 4.0 (Rao *et al.*, 2001). Over a period of time there would be a reduction in phosphate and/or protons would eventually enter the cell and this would subsequently lead to a decrease in internal pH, causing induced expression of bioluminescence under control of P*pd*. Thus, a drop in internal pH regulation is required for induction of Rv2531c but not for the acid tolerance response. This activation of P*pd* coincides with a decrease in survival of *M. smegmatis*. Evidence supporting this finding demonstrated that amino acid decarboxylases were not activated until cell growth ceased and the optimal time at which this occurred in *E. coli* was after sixteen hours (Gale, 1946). This suggests that these amino acid decarboxylases may have evolved as mechanisms to enable the cell to continue to exist in an environment rendered unfavourable by its metabolism during the early stages of growth (Gale, 1946). Furthermore, the induction of the *cad*A gene product in *E. coli* takes over thirty minutes whilst habituation occurs in seven to ten minutes and only three minutes of protein synthesis is required demonstrating that Rv2531c may not be necessarily crucial in the induction of acid tolerance (Rowbury and Goodson, 1993).

6.4.4. Is P*phoPR* acid-inducible in *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG?

PhoP and PhoQ is a two-component regulatory system proved to be important for tolerance to inorganic acid stress (Soncini *et al*, 1995). PhoPQ of *S. typhimurium* is synonymous to PhoPR (a two-component regulator) of *M. tuberculosis*. PhoQ is an inner membrane sensor-kinase protein and PhoP functions as a transcriptional activator. PhoP has also been identified as an acid shock protein (ASP29) and is involved in the acid tolerance response of *S. typhimurium* (Foster, 1991). The promoter for the gene *phoPR* was studied in order to test the hypothesis that it was acid-inducible and that it was involved in the acid tolerance response. To test this, *M. smegmatis* (pJS13) and BCG (pJS13) were exposed to acidic external pH and observed for increased expression of the *luxAB* genes downstream of the promoter P*phoPR*, with hydrochloric acid (HCl) and phosphoric acid (H_3PO_4).

M. smegmatis (pJS13) was grown at pH of 7.6 and subsequently exposed to an adaptive pH of 5.0 or pH 4.5 with H_3PO_4 and observed for increased expression of *luxAB*. Low

levels of bioluminescence were detected during the first eight hours of exposure from *M. smegmatis* (pJS13). These levels were significantly ($P < 0.05$) different with adapted cultures at pH 5.0 or pH 4.5 compared with unadapted cultures from two hours to eight hours. After eight hours, bioluminescence appeared to increase considerably. Approximately 154-fold increase in light production was seen at pH 5.0 and 31-fold increase at pH 4.5, after exposure for forty eight hours after cultures were dropped from pH 7.6. However, statistically only pH 4.5 adapted cultures were different at twenty four to thirty two hours. Thus, indicating that P*phoPR* was induced under acidic external conditions throughout most of the experiment. No light was detected after twenty four hours from the unadapted cultures.

M. smegmatis (pJS13) was also tested using hydrochloric acid as the acidulant. When cultures were exposed at an adaptive pH of 4.5 or pH 4.0, significantly increased levels of bioluminescence were seen compared with unadapted cultures. Cultures adapted at pH 4.5 and pH 4.0 produced significantly high levels of bioluminescence from two to twenty four hours and from three to twenty four hours respectively. Approximately 62-fold increase in light production was seen with pH 4.5 adapted cultures and approximately 77-fold increase for pH 4.0 adapted cultures after exposure for twenty four hours. Although bioluminescence appeared to be considerably high at thirty two and forty eight hours for the adapted cultures, there was no significant ($P > 0.05$) difference compared with unadapted cultures. Furthermore, no light was produced from the unadapted cultures after twenty four hours. Overall, this demonstrated that P*phoPR* was also inducible under acidic external conditions with HCl as the acidulant.

With *M. smegmatis* the expression studies indicated that P*phoPR* expression at the adaptive pH did coincide with an acid tolerance response. *M. smegmatis* (pJS13) had enhanced survival at a lethal pH of 2.5 over six hours following exposure to an adaptive pH and induced expression of *lux* downstream of P*phoPR* also occurred. This suggests that *phoPR* is involved in the acid tolerance response of *M. smegmatis.* These data also indicate that induction of P*phoPR* is relatively long-term under acidic external conditions with hydrochloric and phosphoric acid since induced expression of *lux* was observed at or after twenty four hours exposure.

Unlike *M. smegmatis,* when BCG (pJS13) was grown at pH 7.6 and subsequently exposed at pH 4.5 or pH 4.0 with HCl as the acidulant and pH 6.6 or pH 5.0 with H_3PO_4 as the acidulant, no expression of the *lux* reporter occurred at any time point. Also, no *lux* expression was observed from cultures exposed at pH 7.6. No light was detected from BCG (pJS13) at any time.

Use of the *V. harveyi luxAB* genes could not account for the absence of light production since the *lux* genes are known to function in BCG. A mycobacterial promoter (*hsp*60) placed upstream of the *V. harveyi luxAB* genes (pSMT1) was transformed into BCG and expression of *lux* was detected with the production of high levels of light from BCG (Snewin *et al.*, 1999). In addition, the plasmid pSMT1 has also been used in our work, pSMT1 was transformed into BCG and light was detected when colonies were exposed to decanal and overlaid with Cronex film. This indicates that *lux* is effective as a reporter system in BCG.

No expression of *lux* was observed at any pH with either acidulant. This perhaps suggests that either P*phoPR* is not acid-inducible in BCG or it is possible that the transcriptional machinery (RNA polymerase) of BCG does not recognise the *M. tuberculosis* promoter P*phoPR* whereas the *M. smegmatis* transcriptional machinery does. One study has done a comparative assessment of the ability of RNA polymerases from *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* to recognise mycobacterial promoters from other mycobacteria (Bashyam *et al.*, 1996). Vectors were generated that incorporated chloramphenicol acetyltransferase (CAT) and a *M. tuberculosis* promoter. Two vectors each carrying one *M. tuberculosis* promoter was transformed into *M. smegmatis* and BCG. CAT specific activity was measured from the recombinant mycobacteria and activity was found to be comparable in *M. smegmatis* and BCG (Bashyam *et al.*, 1996). This demonstrated that both *M. smegmatis* and BCG could recognise *M. tuberculosis* promoters with similar efficiency.

A further report has demonstrated the efficiency of *M. tuberculosis* promoters in BCG. PhoPR has similarity to the two-component regulatory system, MtrA and MtrB (Via *et al.*, 1996). The *mtrA* isolated from *M. tuberculosis* was cloned into a vector containing the gene *gfp* coding for green fluorescent protein (*mtrA-gfp*) to observe expression (Via *et al.*, 1996). *mtrA-gfp* was transferred into BCG and expression was monitored upon

entry into macrophages. The *mtrA* promoter was induced upon entry into macrophages demonstrating that *mtrA* from *M. tuberculosis* when present in BCG could be recognised and induced (Via *et al.*, 1996).

However, when mycobacterial promoters were assessed in *M. smegmatis* and BCG, results demonstrated that RNA polymerases from *M. smegmatis* and BCG did not share the same specificity (Timm *et al.*, 1994). The activities of the promoters in *M. smegmatis* and BCG expressing β-galactosidase varied considerably. The relative strengths of the promoters were not the same for both hosts; some of the promoters were more powerful in *M. smegmatis* than in BCG whilst others were more powerful in BCG (Timm *et al.*, 1994). Nevertheless, these results do suggest that the transcriptional machinery of *M. smegmatis* and BCG are different.

Additionally, lack of observable expression could have been down to experimental procedure, such as timing of the experiments. Cultures were measured for the production of light at time zero and six hours, possibly cultures may have needed to be measured over every hour.

The role of PhoPQ on acid tolerance in *S. typhimurium* has been demonstrated through mutations in these genes (Bearson *et al.*, 1998). In particular, *phoP* mutants failed to induce four acid shock proteins confirming a role for this regulator in acid tolerance (Bearson *et al.*, 1998). Further evidence demonstrates that PhoPQ dependent systems protect against inorganic acid stress. The inorganic acid used was not cited. A *phoP* mutant was tested for inorganic acid tolerance and was compared to a *phoP* positive cell. Cultures were grown at pH 7.7 and adapted at pH 4.4 for one hour before being subjected to pH 3.0 for one hour. Unadapted cell cultures were adjusted directly to pH 3.0. The adapted *phoP* mutant was found to be extremely acid sensitive, with survival at approximately 0.03% compared with approximately 70% survival for the *phoP* positive cell (Bearson *et al.*, 1998). In *M. smegmatis,* P*phoPR* did behave fairly similarly to *S. typhimurium*, in that, P*phoPR* was involved in the acid tolerance response.

The expression of *lux* downstream of P*phoPR* was seen using phosphoric and hydrochloric acid as the acidulants, as was acid tolerance (Chapter 4., Figures 4.26., 4.27., 4.21., and 4.22., and Tables 4.11., 4.12., 4.6., and 4.7). Thus, the acid tolerance

response and expression studies overlap. The acid tolerance response of *M. smegmatis* (pJS13) and expression of *lux* was similar with both acidulants, except that cultures adapted at pH 4.5 and acidified with phosphoric acid were induced for over thirty two hours compared with only twenty four hours at pH 4.5 with hydrochloric acid as the acidulant (Chapter 4., Figure 4.26 and 4.27).

6.4.5. Expression of P*pd* or P*phoPR* at the lethal pH in *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG.

According to the model of the acid tolerance response in *Salmonella* the acid shock proteins that protect and/or repair cell damage are produced in two ways. Bacteria exposed to the adaptive pH of 5.8 (pre-acid shock) induce a pH homeostasis system that allows acid shock protein synthesis at external pH values that normally prevents protein synthesis, that is at pH 3.3. Also, bacteria exposed to pH 4.3 (post-acid shock) induce acid shock proteins that allow survival at pH 3.3 (Foster, 1993). Therefore, since expression can be seen at the lethal pH, expression of *lux* downstream of P*pd* or P*phoPR* was measured at the lethal pH with recombinant *M. smegmatis* and BCG.

No expression of *lux* downstream of P*phoPR* occurred at pH 2.5 and pH 3.0 in *M. smegmatis* (pJS13) and BCG (pJS13) respectively following adaptation. Lack of expression could be due to internal changes in pH, which are sufficient to stop light production but not to kill the cell. Thus, we see an acid tolerance response but no *lux* expression. Internal pH was not measured in our work; future experiments could involve measuring changes in internal pH. Measurement of internal pH can be done using two types of probes, weak acids and bases and pH sensitive fluorescent probes (Booth 1985). The important properties of a molecular probe are firstly that the neutral species of the probe should be permeant. Secondly, the probe should not be actively transported, metabolised or interfere with cellular metabolism and thirdly, it should not interact and bind non-specifically to cell components (Booth, 1985). One method uses the distribution of weak acids and bases across a membrane to determine internal pH (Padan and Schuldiner, 1986). Essentially this method is based on the assumption that the uncharged form of the weak acid and base is freely permeable through the membrane whilst the ionised form is almost impermeable. Consequently, the undissociated form of the acid will enter the membrane, dissociate inside and become impermeable (Padan and Schuldiner, 1986).

The fluorescent probe carboxyfluorescein diacetate has been used to measure internal pH; it is split by esterases present in the cytoplasm to release the fluorescent probe, carboxyfluorescein, into the cytoplasm (Booth 1985). The fluorescent intensity of carboxyfluorescein is pH dependent and therefore can be used as an indicator of cytoplasmic pH. However, the main problem with this probe is the high permeability of the cytoplasmic membrane to carboxyfluorescein thus limiting its usefulness (Booth 1985).

M. smegmatis (pJS12) and *M. smegmatis* (pJS13) generated light when exposed to the adaptive pH, but no light was detected at the lethal pH of 2.5. Since induced expression of *lux* was seen after exposure at the adaptive pH for twenty four hours, cultures may have required a longer exposure at the adaptive pH for expression of *lux* at the lethal pH. *M. smegmatis* (pJS12) was adapted at pH 4.5 and *M. smegmatis* (pJS13) was adapted at pH 5.0 or pH 4.5 for twenty four hours before exposure to the lethal pH of 2.5 with phosphoric acid or hydrochloric acid as the acidulants. When *M. smegmatis* (pJS12) was tested with H_3PO_4, high levels of light were detected at pH 2.5 at time zero from cultures adapted at pH 4.5 compared with unadapted cultures. These results further indicated that *luxAB* gene expression was induced under the control of P*pd* when cultures were incubated for twenty four hours at the adaptive pH. However, with both strains bioluminescence declined at pH 2.5 over six hours suggesting that *luxAB* expression could not be maintained at this pH with H_3PO_4 or HCl. Except for *M. smegmatis* (pJS13) adapted at pH 4.5, significantly increased expression of *lux* did occur at the one hour time point only. Overall, adaptation for twenty four hours did not increase the expression of the *lux* system downstream of P*pd* and P*phoPR* at pH 2.5. Thus, the P*pd* and P*phoPR* promoters were not inducible at pH 2.5.

Lack of *lux* expression at the lethal pH of 2.5 with *M. smegmatis* and at pH 3.0 with BCG could also be due to changes in metabolism as a consequence of changes in internal pH. Limitation in substrate availability, for example, $FMNH_2$ that is necessary for the bacterial luciferase reaction could result in loss of light production. A reduction in internal pH could also result in the enzyme luciferase becoming unstable and thus leading to loss of light. The optimum pH for luciferase is pH 6.8 in *P. fischeri* (Hastings *et al.*, 1969). The range of pH at which luciferase will remain stable and function

adequately is unknown; no studies have demonstrated this phenomenon. However, when the expression of *lux* downstream of P*phoPR* was measured from *M. smegmatis* (pJS13) exposed at pH 3.0, extremely high expression was seen and furthermore light production increased over six hours. This demonstrated that luciferase was stable at these lower pH values and thus, enzyme instability and lack of $FMNH_2$ could not account for absence of *lux* expression in BCG. This explanation for *lux* is still possible for *M. smegmatis* at pH 2.5.

As mentioned earlier, when *M. smegmatis* (pJS13) was exposed to pH 3.0 following adaptation for two hours at pH 5.0 or pH 4.5 with H_3PO_4, induction of expression of *lux* downstream of P*phoPR* was seen after three hours and after three to fours hours, respectively. However, with *S. typhimurium*, *phoP* was induced immediately for one hour following a pH shift to pH 3.0 (Bearson *et al.*, 1998). In *M. smegmatis* no immediate observable induction of P*phoPR* was seen. Furthermore, *M. smegmatis* (pJS13) exposed to a lethal pH of 2.5 survived significantly better over six hours compared with exposure at pH 3.0 and expression of *lux* was observable at two hours onwards at the adaptive pH. This suggests that in *M. smegmatis* the occurrence of the adaptive pH was more important in providing protection against pH 2.5 than at pH 3.0. At pH 3.0, mycobacteria may use other constitutive mechanisms to deal with changes in pH.

Cultures of *M. smegmatis* (pJS12) and *M. smegmatis* (pJS13) did not survive significantly better at pH 2.5 following adaptation at pH 4.5 over twenty four hours compared with unadapted cultures using H_3PO_4 or HCl. Thus, adaptation over twenty four hours does not lead to an acid tolerance response that provides protection at pH 2.5. It is possible that if there is a delay encountering lethal acid levels following adaptation, some of the proteins are no longer synthesised and the cell subsequently becomes vulnerable to low pH. In *S. typhimurium* the loss of acid shock proteins after 20 to 30 minutes of pH 4.4 acid shock coincided with an inability to survive challenge at a lethal pH of 3.3 (Foster, 1993).

6.4.6. Knockout mutation of the Rv2531c gene in *Mycobacterium smegmatis*.

An Rv2531c gene knockout was constructed in *M. smegmatis* to determine the response of the mutant to acid stress. A positive PCR for the hygromycin gene confirmed integration of pJS23. It should be noted however, that the dot-blot described in section 5.4 only indicates cross-hybridisation of the probe with total genomic DNA. A Southern blot using Rv2531c as a probe against *M. smegmatis* genomic DNA would have shown whether the level of nucleic acid identity between *M. smegmatis* and *M. tuberculosis* was satisfactory to generate a knockout in *M. smegmatis*. Furthermore, it could be possible that the method used for the selection of mutant would have not allowed the differentiation of spontaneous hygromycin resistant mutants from possible double-crossovers. If done differently, the first selection would have been on plates containing kanamycin and hygromycin to select for integration of the construct. The colonies would then have been replica-plated onto plates containing hygromycin and sucrose to select for possible double-crossover events.

There are several counterselectable markers available for use in bacteria for the positive selection of mutants (Reyrat *et al.*, 1998). The *sacRB* gene, a modified derivative of the *Bacillus subtilis sacB* gene encodes levansucrase that confers sucrose sensitivity. Its expression is lethal to mycobacteria in the presence of ten percent sucrose (Pelicic *et al.*, 1996a; 1996b). This gene is very efficient in *M. smegmatis* for the selection of double recombination events (Pelicic *et al.*, 1996b). Thus, the *sacRB* gene was used as a selectable marker in experiments with Rv2531c. In our work, all the transformants isolated had undergone a double recombination event. These transformants were hygromycin resistant, kanamycin sensitive and sucrose resistant. Thus, the *sacRB* gene was very efficient for the selection of the desired mutants.

Experiments to test for an acid tolerance response were repeated with the Rv2531c mutant *M. smegmatis* (pJS12-dc). A comparison of *M. smegmatis* (pJS12-dc) with *M. smegmatis* (pJS12) (wild-type) demonstrated that Rv2531c was involved in the acid tolerance response of *M. smegmatis* when phosphoric acid was used as the acidulant, since *M. smegmatis* (pJS23-dc) adapted at a pH of 4.5 survived significantly worse at pH 2.5 compared with *M. smegmatis* (pJS12). However, Rv2531c was not crucial when hydrochloric acid was the acidulant since *M. smegmatis* (pJS12-dc) did not survive significantly worse than *M. smegmatis* (pJS12).

No studies have compared the acid tolerance responses of bacteria to different inorganic acids, such as, hydrochloric and phosphoric acid. However, studies have been done to compare acid tolerance responses to different organic acids. In one study three organic acids were compared, acetic, citric and malic acid. *E. coli* O157:H7 cells were adapted or unadapted and subsequently exposed to low pH and tolerance to organic acid was measured (Deng *et al.*, 1999). *E. coli* O157:H7 was less tolerant to acetic acid than to citric or malic acid, thus the order of inhibition was acetic>citric>malic acid demonstrating that acid tolerance was affected by the type of acidulant (Deng *et al.*, 1999).

The *V. cholerae cadA* gene (lysine decarboxylase) was shown to be essential for the acid tolerance response to inorganic acid (hydrochloric acid) challenge (Merrell and Camilli, 1999). In our work, the putative decarboxylase Rv2531c was not crucial for the acid tolerance response using hydrochloric acid as the acidulant. *V. cholerae* adapted for one hour at pH 5.7 completely survived acid shock at pH 4.5 over one hour compared with unadapted cells grown at pH 7.0, which were rapidly killed demonstrating *V. cholerae* was able to mount an ATR using hydrochloric acid. In addition, a *cad*A mutant was tested for an ATR. These adapted cells were unable to mount an acid tolerance response and showed less than 10% survival at one hour compared with 100% survival for the wildtype demonstrating that *cad*A was involved in the acid tolerance response of *V. cholerae* when using hydrochloric acid (Merrell and Camilli, 1999). An acid tolerance response was also seen with *S. typhimurium*, cells grown at pH 7.7 and adapted at pH 4.4 for one hour subsequently survived acid challenge at pH 3.0 over ninety minutes. The acidulant used was not cited. A *S. typhimurium cad* mutant was unable to induce an effective acid tolerance response when adapted and acid challenged at pH 3.0.

*cad*A mutants were tested for acid sensitivity in *E. coli* with hydrochloric acid as the acidulant (Rowbury and Goodson, 1993). Cells were not affected in their ability to acquire acid tolerance at pH 5.0 and also they did not have increased acid sensitivity when grown at pH 7.0. The *cad*A mutant grew well and its growth was shown to be better than its parent, it was suggested that CadA might support growth at acid pH (Rowbury and Goodson, 1993). Viability of *M. smegmatis* (pJS12-dc) also appeared to be better than the wildtype using hydrochloric acid as the acidulant. Thus,

decarboxylase is not crucial to the acid tolerance response of *E. coli* and *M. smegmatis* with hydrochloric acid as the acidulant. Another study has also shown that *cad*A knockout mutants are protected to some extent from acid exposure. A *S. typhimurium cad*A mutant demonstrated some acid tolerance to pH 3.0 following adaptation at pH 4.4, but was less than the wild type (Park *et al.*, 1996). In addition, the *V. cholerae cad*A mutant when adapted was still found to have some protection when acid shocked (Merrell and Camilli, 1999).

6.5. Future work.

'Proteome' refers to all the proteins expressed by a genome and proteomics involves the identification and analysis of proteins. Proteomics has two specific disciplines, classical and functional proteomics. Classical proteomics involves the study of two or more proteomes from cells that have been differentially treated. These are separated and visualised by two-dimensional gel electrophoresis and proteins that differ in abundance between the gels are identified by mass spectrometry. Functional proteomics involves the isolation of a subset of proteins by a specific cell at a certain point in time. It allows the study of how proteins interact with other cellular components in order to determine protein function of each characterised protein using bioinformatics.

The proteins in a cell can change considerably as genes are turned on and off in response to its environment. Proteomics is useful in order to determine how a cell responds to a signal at the protein level and also provides information about post-translational modifications. Future work could be done to identify and analyse proteins from bacteria during the acid tolerance response, for example, two populations of cells are used, one is adapted to acid stress and the other unadapted. The proteins for the two populations are then analysed to compare the two groups. Proteomics could be used to provide information to which proteins are up- or down- regulated in response to these changes. Three main steps are involved in proteome research: separation of individual proteins by two-dimensional polyacrylamide gel electrophoresis. Identification by mass spectrometry or N-terminal sequencing of individual proteins recovered from the gel and finally storage, manipulation, and comparison of the data using bioinformatics. The expression of acid shock proteins has been analysed by two-dimensional electrophoresis (Heyde and Portalier, 1990; Foster, 1993; Karem *et al.*, 1994; Davis *et al.*, 1997).

Proteomics is one approach in identifying and analysing proteins. In order to analyse the expression of genes the second approach forward would be to use microarray. The complete sequencing and annotation of the *M. tuberculosis* genome has been done. As a result, Dr. Philip D. Butcher at St George's Hospital Medical School has recently established a microarray facility (http://www.sghms.ac.uk/depts/medmicro/bugs/). This is a powerful and developing tool that permits the comparative analysis of gene expression. This technology could be used to observe the expression of genes in mycobacteria as a result of environmental stress. The microarray technology can be used for mRNA expression studies to compare two populations of cells to determine which genes are activated and which genes are repressed. The cells are grown under the two conditions, for example, adapted to environmental stress or unadapted, allowing for genes to become activated or inactivated. The mRNA is extracted from the cells and cDNA is made that is labelled using two fluorescently labelled probes. The two fluorescently labelled probes containing the two populations of cDNA are then mixed together and hybridised to the DNA microarray (DNA chip) containing the entire sequence of the genome. The microscope slide containing the microarray is scanned with a red and green laser to detect the bound cDNA. The computer creates a merged image of the two scans, and further analysis determines whether genes are expressed only in one population of cells or both.

APPENDIX 1.

Table 1.1. Genes regulated by pH and/or involved in the acid tolerance response.

Definition	Accession number	Reference
E. coli arginine decarboxylase (*adi*)	M93362	Stim and Bennett, 1993.
E. coli ornithine decarboxylase (*dcor*)	P21169	Blattner *et al.*, 1997.
E. coli lysine decarboxylase (*cadA*)	M67452	Leveque *et al.*, 1991. Watson *et al.*, 1992.
E. coli transport protein (*cadB*)	M67452	Leveque *et al.*, 1991. Watson *et al.*, 1992.
E. coli regulator (*cadC*)	M67452	Leveque *et al.*, 1991. Watson *et al.*, 1992.
ORF-1 *M. smegmatis*	-	Gordon, S. 1995.
S. typhimurium mouse virulence gene A (*mviA*)	U53173	Benjamin *et al.*, 1996.
E. coli glutamate decarboxylase-alpha (*gadA*)	M84024	Smith *et al.*, 1992.
E. coli glutamate decarboxylase-beta (*gadB*)	M84025	Smith *et al.*, 1992.
E. coli isocitrate dehydrogenase (*icd*)	AAF97071	Reid *et al.*, 2000.
E. coli pyruvate dehydrogenase E1 component (*odp*1)	P06958	Stephens *et al.*, 1983
E. coli adenylosuccinate lyase (*purB*)	M74924	He *et al.*, 1992.
E. coli ferric uptake regulator (*fur*)	P06975	Schaeffer *et al.*, 1985. Bagg and Neilands, 1987b.
E. coli Glucose-6-phosphate isomerase (*gpi*)	P11537	Froman *et al.*, 1989.
Hypothetical 28.9 Kda protein in osmY-deoc intergenic region (*osmY*)	P39408	Burland *et al.*, 1995.
3-oxoacyl-[acyl-carrier-protein] synthase II (beta) (*fab*F) also known as Ketoacyl-ACP synthase II (*kas*II).	P39435	Siggaard-Andersen *et al.*, 1994. Magnuson *et al.*, 1995.
E. coli regulatory protein of adaptive response (*ada*)	M10211	Nakabeppu *et al.*, 1985.
S. typhimurium virulence (*pagD*)	AAA82994	Gunn *et al.*, 1995.
S. typhimurium virulence (*pagC*)	M55546	Pulkkinen and Miller, 1991.
E. coli sigma factor (*rpoS*)	AF002209	Ferreira *et al.*, unpuplished. Ferreira *et al.*, 1997.

Figure 1.1. Blast search of *E. coli adi* gene (arginine decarboxylase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY159

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Query:     139 LPPLFSALMKYS--DIHEYSWAAPGHQGGVGFTKTPAGRFYHD---YYGENLFRTDMGIE 193
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Query:     194 RTSLGSLLDHTGAFGESEKYAARVFGADRSWSVVVGTSGSNRTIMQACMTDNDVVVVDRN 253
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Query:     312 -KPSYCVVTNCTYDGVCYNAKEAQD--LLEKTSDRLHFDEAWYGYARFNPIYADHYAM 366
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Query:     437 MMDGNSGLSLTQEVIDEAVDFRQAMAR 463
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Figure 1.2. Blast search of *E. coli dcor* gene (ornithine decarboxylase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY159

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Query:     241 HGALIQAGATPVYLEASRNP-FGFIGGIDAHCFNEEYLRQQIRDVAPEKADLPRPYRLAI 299
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DPTRLTLFIGATGMNGYDFREKILMERFGIQINKTSINSVLLIFTIGVTWSSVHYLLDVLRRVAIDL
DRSQKAASGADLALHRRHVEEITQDLPHLPDFSEFDLAFRPDDASSFGDMRSAFYAGYEEADRE
YVQIGLAGRRLAEGKTLVSTTFVVPYPPGFPVLVPGQLVSKEIIYFLAQLDVKEIHGYNPDLGLSV
FTQAALARMEAARNAVATVGAALPAFEVPRDASALNGTVNGDSVLQGVAEDA"

Figure 1.3. Blast search of *E. coli cadA* gene (lysine decarboxylase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY159

```
  Minus Strand HSPs:

 Score = 196 (69.0 bits), Expect = 2.2e-12, Sum P(2) = 2.2e-12
 Identities = 63/219 (28%), Positives = 95/219 (43%), Frame = -3

Query:       58 DWDKYNLELCEEISKMNENLPLYAFANTYSTLDVSLNDLRLQISFFEYALGAAEDIANKI 117
                DW    +E  E I ++  ++ LY    T  ++     D   +   ++       D+ +
Sbjct: 2857101 DW----VECAEWIRELRPHIDLYLL--TDESIAAETQD---EPDVYDRTFYRLNDVTDLH 2856949

Query:      118 KQTTDEYINTILPPLTKALFKYVREGKYTF-CTPGHMGGTAFQKSPVGSLFYDFFGPNTM 176
                        N    P   AL  Y       F   P   G + F    +  +  +F+G N
Sbjct: 2856948 STVLAGLRNRYATPFFDALRAYAAAPVGQFHALPVARGASIFNSKSLHDMG-EFYGRNIF 2856772

Query:      177 KSDISISVSELGSLLDHSGPHKEAEQYIARVFNADRSYMVTNGTSTANKIVGMYSAPAGS 236
                ++ S +   L SLLD  G  K A    A  +NA+++Y VTNGTSTANKIV       G
Sbjct: 2856771 MAETSTTSGGLDSLLDPHGNIKTAMDKAAVTWNANQTYFVTNGTSTANKIVVQALTRPGD 2856592

Query:      237 TILIDRNCHKSLTHLMMMSDVTPIYFRP-TRNAYGILGGIP 276
                +LIDRNCHKS  + ++++   P+Y        Y I G +P
Sbjct: 2856591 IVLIDRNCHKSHHYGLVLAGAYPMYLDAYPLPQYAIYGAVP 2856469
```

CDS complement(14986..17829)
/db_xref="SPTREMBL:P95022"
/note="Rv2531c, (MTCY159.25). Possible adi, ornithine/arginine decarboxylase, similar to eg DCOR_ECOLI P21169 ornithine decarboxylase.

/gene="*adi*"
/product="Adi"
/translation=

"MNPNSVRPRRLHVSALAAVANPSYTRLDTWNLLDDACRHLAEVDLAGLDTTHDVARAKRLM
DRIGAYERYWLYPGAQNLATFRAHLDSHSTVRLTEEVSLAVRLLSEYGDRTALFDTSASLAEQE
LVAQAKQQQFYTVLLADDSPATAPDSLAECLRQLRNPADEVQFELLVVASIEDAITAVALNGEIQ
AAIIRHDLPLRSRDRVPLMTTLLGTDGDEAVANETHDWVECAEWIRELRPHIDLYLLTDESIAAE
TQDEPDVYDRTFYRLNDVTDLHSTVLAGLRNRYATPFFDALRAYAAAPVGQFHALPVARGASIF
NSKSLHDMGEFYGRNIFMAETSTTSGGLDSLLDPHGNIKTAMDKAAVTWNANQTYFVTNGTST
ANKIVVQALTRPGDIVLIDRNCHKSHHYGLVLAGAYPMYLDAYPLPQYAIYGAVPLRTIKQALL
DLEAAGQLHRVRMLLLTNCTFDGVVYNPRRVMEEVLAIKPDICFLWDEAWYAFATAVPWARQ
RTAMIAAERLEQMLSTAEYAEEYRNWCASMDGVDRSEWVDHRLLPDPNRARVRVYATHSTHK
SLSALRQASMIHVRDQDFKALTRDAFGEAFLTHTSTSPNQQLLASLDLARRQVDIEGFELVRHVY
NMALVFRHRVRKDRLISKWFRILDESDLVPDAFRSSTVSSYRQVRQGALADWNEAWRSDQFVL
DPTRLTLFIGATGMNGYDFREKILMERFGIQINKTSINSVLLIFTIGVTWSSVHYLLDVLRRVAIDL
DRSQKAASGADLALHRRHVEEITQDLPHLPDFSEFDLAFRPDDASSFGDMRSAFYAGYEEADRE
YVQIGLAGRRLAEGKTLVSTTFVVPYPPGFPVLVPGQLVSKEIIYFLAQLDVKEIHGYNPDLGLSV
FTQAALARMEAARNAVATVGAALPAFEVPRDASALNGTVNGDSVLQGVAEDA"

Figure 1.4. Blast search of *E. coli cadB* gene (transport protein) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY39

```
  Minus Strand HSPs:

 Score = 233 (82.0 bits), Expect = 1.2e-17, P = 1.2e-17
 Identities = 117/440 (26%), Positives = 190/440 (43%), Frame = -1

Query:     5 KKIGLFACTGVVAGNMMGSGI--ALLPANLXXXXXXXXXXXXXXXXXXMSLAYVYARLAT 62
             +++GL     +  G+M+G+GI  AL PA                      + A   ARLA
Sbjct: 21841 RRLGLLDAVVIGLGSMIGAGIFAALAPAAYAAGSGLLLGLAVAAVVAYCN-AISSARLAA 21665

Query:    63 KNPQQGGPIAYAGEISPAFGFQTGVLYYHANWIGNLAIGITA---VSYLSTFFPVLNDPV 119
             + P  GG   Y G +    G   G   Y A W     +G TA      L+  F V   P
Sbjct: 21664 RYPASGGTYVY-GRMR--LGDFWG---YLAGW--GFVVGKTASCAAMALTVGFYVW--PA 21515

Query:   120 PAGIACIAIVWVFTFVNMLG---GTWVSRLTTIGLVLVLIPVVMTAIVGWHWFDAATYAA 176
              A    +A+V   T VN  G     W++R + + +VLV++  V+ A  G      A   A
Sbjct: 21514 QAHAVAVAVVVALTAVNYAGIQKSAWLTR-SIVAVVLVVLTAVVVAAYG----SGAADPA 21350

Query:   177 NWNTADTTDGHA--IIKSILLCLWAFVGVESAAVSTGMVKNPKRTVPLATMLGTGLAGIV 234
               +     D H   ++++  L  +AF G    A     V++P RT+P A  L  G+   V
Sbjct: 21349 RLDIG--VDAHVWGMLQAAGLLFFAFAGYARIATLGEEVRDPARTIPRAIPLALGITLAV 21176

Query:   235 Y-IAATQVLSGMYPSSVMAASGAPFAISASTILG-NWAAPLVSAFTAFACLTSLGSWMML 292
             Y + A  V++ + P   +A + AP +  A  + G NW  P+V    A A L SL + ++
Sbjct: 21175 YALVAVAVIAVLGPQR-LARAAAPLS-EAMRVAGVNWLIPVVQIGAAVAALGSLLALILG 21002

Query:   293 VGQAGVRAANDGNFPKVYGEVDSN-GIPKKGLLLAAVKMTALMILITLMNSAGGKASDLF 351
             V +  + A D + P+    V     +P +  L+    + AL     +  + G  +   F
Sbjct: 21001 VSRTTLAMARDRHLPRWLAAVHPRFKVPFRAELVVGAVVAALAATADIRGAIGFSS---F 20831

Query:   352 GELTGIAVLLTMLPYFYSCVDLIRFEGVNIRNFVSLICSVLGCVFCFIALMGASSFELAG 411
             G L   A+         S + L   EG   R  + L+  ++GCV    AL  +S    AG
Sbjct: 20830 GVLVYYAIANA------SALTLGLDEG-RPRRLIPLV-GLIGCVVLAFALPLSSV--AAG 20681

Query:   412 TFIVSLIILMFYARKMHERQSHSMDN-HTASNAH 444
               ++ + +  +  R++  R++   D+  T  + H
Sbjct: 20680 AAVLGVGVAAYGVRRIITRRARQTDSGDTQRSGH 20579
```

CDS complement(20564..21886)
/db_xref="SWISS-PROT:Q10858"
/note="Rv1999c, (MTCY39.19). Transport protein.

/gene="Rv1999c"
/product="hypothetical protein Rv1999c"
/translation=

"MRRPLDPRDIPDELRRRLGLLDAVVIGLGSMIGAGIFAALAPAAYAGSGLLLGLAVAAVVAYC
NAISSARLAARYPASGGTYVYGRMRLGDFWGYLAGWGFVVGKTASCAAMALTVGFYVWPAQ
AHAVAVAVVVALTAVNYAGIQKSAWLTRSIVAVVLVVLTAVVVAAYGSGAADPARLDIGVDA
HVWGMLQAAGLLFFAFAGYARIATLGEEVRDPARTIPRAIPLALGITLAVYALVAVAVIAVLGPQ
RLARAAAPLSEAMRVAGVNWLIPVVQIGAAVAALGSLLALILGVSRTTLAMARDRHLPRWLAA
VHPRFKVPFRAELVVGAVVAALAATADIRGAIGFSSFGVLVYYAIANASALTLGLDEGRPRRLIP
LVGLIGCVVLAFALPLSSVAAGAAVLGVGVAAYGVRRIITRRARQTDSGDTQRSGHPSAT"
MTCI125

```
  Minus Strand HSPs:

 Score = 183 (64.4 bits), Expect = 3.7e-12, P = 3.7e-12
 Identities = 80/337 (23%), Positives = 144/337 (42%), Frame = -2

Query:    71 IAYAGEI-SPAFGFQTGVLYYHANWIGNLAIGITAVS-YLSTFFPVLNDPVPAGIACIAI 128
             + +A ++  PA GF  G  Y+ A W+      + A++ Y   ++P L   VPA +    I
Sbjct: 26781 VDFAADLRGPAAGFFVGWSYWFA-WVVTGIADLVAITGYARFWWPGLPIWVPALVTVALI 26605

Query:   129 VWVFTF-VNMLGGT--WVSRLTTIGLV-LVLIPVVMTA--IVGWHWFDAATYAANWNTAD 182
             + V  F V   G    W + +    +V L+ +  ++ A   V  H   A T    WN
Sbjct: 26604 LAVNLFSVRHFGELEFWFALIKVAAIVCLIAVGAILVATNFVSPHGVHA-TIENLWNDNG 26428

Query:   183 --TTDGHAIIKSILLCLWAFVGVESAAVSTGMVKNPKRTVPLATMLGTGLAGIVYIAATQ 240
                T    ++    +  +A++GVE    +     +P+RT+P A         + YI A
Sbjct: 26427 FFPTGFLGVVSGFQIAFFAYIGVELVGTAAAETADPRRTLPRAINAVPLRVAVFYIGALL 26248

Query:   241 VLSGMYPSSVMAASGAPFAISASTILGNWAAPLVSAFTAF-ACLTSLGSWMMLVGQAGVR 299
              +  + P    A+  +PF ++  ++ G  AA  V  F    A  +S  S     G+
Sbjct: 26247 AILAVVPWRQFASGESPF-VTMFSLAGLAAAASVVNFVVVTAAASSANSGFFSTGRMLFG 26071

Query:   300 AANDGNFPKVYGEVDSNGIPKKGLLLAAVKMTALMILITLMNSAGGKASDLFGELTGIAV 359
              A++G+ P  + +++  G+P   LLL A     L+  I L+  AG      F  +T ++
Sbjct: 26070 LADEGHAPAAFHQLNRGGVPAPALLLTA---PLLLTSIPLLY-AGRSVIGAFTLVTTVSS 25903

Query:   360 LLTMLPYFYSCVDLIRFEGVNIRNFVSLICSVLG-CVFCFIALM 402
             LL M  +    + + +   + +     +  + G  V C+  L+
Sbjct: 25902 LLFMFVWAMIIISYLVYRRRHPQRHTDSVYKMPGGVVMCWAVLV 25771
```

MTCI125

CDS 1..556
/db_xref="SPTREMBL:O33203"
/note="Rv1704c, (MTCI125.26c). D-serine/D-alanine/glycine transporter.

/gene="*cycA*"
/product="cycA"
/translation=

"MPDDIAAADPTDTQPHLRRDLANRHIQLIAIGGAIGTGLFMGSGRTISLAGPAVMVVYGIIGFFV
FFVLRAMGELLLSNLNYKSFVDFAADLRGPAAGFFVGWSYWFAWVVTGIADLVAITGYARFW
WPGLPIWVPALVTVALILAVNLFSVRHFGELEFWFALIKVAAIVCLIAVGAILVATNFVSPHGVH
ATIENLWNDNGFFPTGFLGVVSGFQIAFFAYIGVELVGTAAAETADPRRTLPRAINAVPLRVAVF
YIGALLAILAVVPWRQFASGESPFVTMFSLAGLAAAASVVNFVVVTAAASSANSGFFSTGRMLF
GLADEGHAPAAFHQLNRGGVPAPALLLTAPLLLTSIPLLYAGRSVIGAFTLVTTVSSLLFMFVWA
MIIISYLVYRRRHPQRHTDSVYKMPGGVVMCWAVLVFFAFVIWTLTTETETATALAWFPLWFVL
LAVGWLVTQRRQSRRSFGFHCQVVGVRQQLGRGMARLAMKIHARPKLRSAVVVEPVSAGEPG
ARRSAKSVRKLASDDSQSAHCPVAVVGLADGGRDPQYHHDGPDR"

Figure 1.5. Blast search of *E. coli cadC* gene (regulator) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY20G9

```
  Plus Strand HSPs:

 Score = 124 (43.7 bits), Expect = 1.3e-05, P = 1.3e-05
 Identities = 30/81 (37%), Positives = 46/81 (56%), Frame = +2

Query:    20 ISRNGRQLTLEPRLIDLLVFFAQHSGEVLSRDELIDNVWKRSIVTN-HVVTQSISELRKS 78
             +S NG  +TL  +  DLL +  ++SG VL+R +LID VW    V +   +   + LR
Sbjct: 15641 VSVNGDTITLPLKEFDLLEYLMRNSGRVLTRGQLIDRVWGADYVGDTKTLDVHVKRLRSK 15820

Query:    79 LKDNDEDSPVYIATVPKRGYKL 100
             + + D  +PV++ TV   GYKL
Sbjct: 15821 I-EADPANPVHLVTVRGLGYKL 15883
```

CDS 1..227
/db_xref="SWISS-PROT:Q11156"
/coded_by="Z77162.1:15209..15892"
/note="Rv0491, (MTCY20G9.17). Response regulator protein.

/gene="*regX3*"
/product="RegX3"
/translation=

"MTSVLIVEDEESLADPLAFLLRKEGFEATVVTDGPAALAEFDRAGADIVLLDLMLPGMSGTDVC
KQLRARSSVPVIMVTARDSEIDKVVGLELGADDYVTKPYSARELIARIRAVLRRGGDDDSEMSD
GVLESGPVRMDVERHVVSVNGDTITLPLKEFDLLEYLMRNSGRVLTRGQLIDRVWGADYVGDT
KTLDVHVKRLRSKIEADPANPVHLVTVRGLGYKLEG"

MTY20B11

```
  Minus Strand HSPs:

 Score = 125 (44.0 bits), Expect = 2.3e-05, Sum P(2) = 2.3e-05
 Identities = 29/86 (33%), Positives = 47/86 (54%), Frame = -2


Query:    18 NQISRNGRQLTLEPRLIDLLVFFAQHSGEVLSRDELIDNVWK-RSIVTNHVVTQSISELR 76
             ++++RNG Q++L P   DLLV  A+   +V +RD L++ VW  R      +V   +  LR
Sbjct: 24449 HKVTRNGEQISLTPLEFDLLVALARKPRQVFTRDVLLEQVWGYRHPADTRLVNVHVQRLR 24270

Query:    77 KSLKDNDEDSPVYIATVPKRGYKLMVP 103
               + + D ++P  + TV   GYK   P
Sbjct: 24269 AKV-EKDPENPTVVLTVRGVGYKAGPP 24192


 Score = 54 (19.0 bits), Expect = 2.3e-05, Sum P(2) = 2.3e-05
 Identities = 12/22 (54%), Positives = 16/22 (72%), Frame = -2

Query:   368 SSPEFT-YARAEKALVDIVRHS 388
             S PE T Y R+E+ALVD+ + S
Sbjct: 20666 SRPEHTGYPRSEEALVDVAKSS 20601
```

CDS 1..228
/db_xref="SPTREMBL:Q50447"
/note="Rv3246c, (MTCY20B11.21c). Response regulator, putative transcriptional activator.

/gene="*mtrA*"
/product="MtrA"
/translation=

"MDTMRQRILVVDDDASLAEMLTIVLRGEGFDTAVIGDGTQALTAVRELRPDLVLLDLMLPGM
NGIDVCRVLRADSGVPIVMLTAKTDTVDVVLGLESGADDYIMKPFKPKELVARVRARLRRNDD
EPAEMLSIADVEIDVPAHKVTRNGEQISLTPLEFDLLVALARKPRQVFTRDVLLEQVWGYRHPAD
TRLVNVHVQRLRAKVEKDPENPTVVLTVRGVGYKAGPP"

Figure 1.6. Blast search of ORF-1 from *M. smegmatis* against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTV025

```
Minus Strand HSPs:

 Score = 958 (337.2 bits), Expect = 7.5e-96, Sum P(2) = 7.5e-96
 Identities = 210/360 (58%), Positives = 245/360 (68%), Frame = -2

Query:      1 TSDGDRQEVSXXXXXXXXXXXXTREPQTIDLDGLGRYRLIGLHPRHGGPQTIVTGLXNSV 60
              T  G R  ++             +R P T+DLDGLGRYR++   P   G   IVTGL
Sbjct: 117851 TGSGSRAALTSTGRSQLERIAGSRTPLTLDLDGLGRYRVLAA-PSRNGHDVIVTGLSMGN 117675

Query:     61 VDDTLLWVLGMFCXXXXXXXXXXXXXXXXXXRRQFAPLARVSATAREVADLELERGEVRL 120
              VD T+L +L +F                    +R  APL RV+ TA EV DL L+RGEV+L
Sbjct: 117674 VDATMLQMLIIFGIVTVIALVAATTAGIVIIKRALAPLRRVAQTASEVVDLPLDRGEVKL 117495

Query:    121 PTPIVPVDPAVXHTEVGQLGTSXNRMLDRIASALSARHASETRVRQFVADASHELRTPLA 180
              P   VP   A   TEVGQLG++ NRMLD IA+ALSAR ASET VRQFVADASHELRTPLA
Sbjct: 117494 PVR-VPEPDANPSTEVGQLGSALNRMLDHIAAALSARQASETCVRQFVADASHELRTPLA 117318

Query:    181 AIRGYTEXAQRKRGELPDDVAHAMSRVESETSRMTQLVEDMLLLARLDAGRPLERDRVEL 240
              AIRGYTE  QR  G+ P+ VAHAMSRV SET R+T+LVED+LLLARLD+GRPLER  V++
Sbjct: 117317 AIRGYTELTQRI-GDDPEAVAHAMSRVASETERITRLVEDLLLLARLDSGRPLERGPVDM 117141

Query:    241 SRLVVDTVSDRHVAGPQHKWSLDLPEDTVVIDGDEARLHQVMANLLXNARTHTPPGTSVT 300
              SRL VD VSD HVAGP H+W+LDLP + VVI GD ARLHQV+ NLL NAR HT PGT VT
Sbjct: 117140 SRLAVDAVSDAHVAGPDHQWALDLPPEPVVIPGDAARLHQVVTNLLANARVHTGPGTIVT 116961

Query:    301 VALSADAEGWVTVAVTDDGPGIPPELLPDVFERFARXDSSRSPREGSTGLGLAIVAAVVQ 360
                LS      V + V D+GPGIP  L  +VFERFAR D+SRS + GSTGLGLAIV+AVV+
Sbjct: 116960 TRLSTGPTH-VVLQVIDNGPGIPAALQSEVFERFARGDTSRSRQAGSTGLGLAIVSAVVK 116784
```

CDS complement(116679..118106)

/note="Rv3764c, (MTV025.112c). Probable histidine protein kinase, part of a two-component regulatory system.

/gene="Rv3764c"

/product="hypothetical protein Rv3764c"

/translation=

"MGITAATEMALRRHLVAQLDNQLGGTSYRSVLMYPEKMPRPPWRHETHNYIRSGPGPRFLDAP
GQPAGMVAAVVSDGTTVAAGYLTGSGSRAALTSTGRSQLERIAGSRTPLTLDLDGLGRYRVLA
APSRNGHDVIVTGLSMGNVDATMLQMLIIFGIVTVIALVAATTAGIVIIKRALAPLRRVAQTASEV
VDLPLDRGEVKLPVRVPEPDANPSTEVGQLGSALNRMLDHIAAALSARQASETCVRQFVADASH
ELRTPLAAIRGYTELTQRIGDDPEAVAHAMSRVASETERITRLVEDLLLLARLDSGRPLERGPVD
MSRLAVDAVSDAHVAGPDHQWALDLPPEPVVIPGDAARLHQVVTNLLANARVHTGPGTIVTTR
LSTGPTHVVLQVIDNGPGIPAALQSEVFERFARGDTSRSRQAGSTGLGLAIVSAVVKAHNGTITVS
SSPGYTEFAVRLPLDGWQPLESSPR"

MTCY10G2

```
  Minus Strand HSPs:

 Score = 604 (212.6 bits), Expect = 4.9e-59, P = 4.9e-59
 Identities = 125/266 (46%), Positives = 181/266 (68%), Frame = -3

Query:     97 PLARVSATAREVADLELERGEVRLPTPIVPVDPAVXHTEVGQLGTSXNRMLDRIASALSA 156
              PL RV+ATA EVA + L    + ++    + P D     + EVG +G + NR+LD +  AL+
Sbjct: 24040 PLRRVAATAAEVATMPLTDDDHQISVRVRPGDTDPDN-EVGIVGHTLNRLLDNVDGALAH 23864

Query:    157 RHASETRVRQFVADASHELRTPLAAIRGYTEXAQRKRGELPDDVAHAMSRVESETSRMTQ 216
              R  S+ R+RQF+ DASHELRTPLAAI+GY E  ++    +LP     +A++R+ESE  RMT
Sbjct: 23863 RVDSDLRMRQFITDASHELRTPLAAIQGYAELTRQDSSDLPPTTEYALARIESEARRMTL 23684

Query:    217 LVEDMLLLARLDAGRPLERDRVELSRLVVDTVSDRHVAGPQHKWSLDLPEDTVVIDGDEA 276
              LV+++LLL+RL  G  LE + ++L+ LV++ V+D  VA P H+W  +LP++ V ++GD A
Sbjct: 23683 LVDELLLLSRLSEGEDLETEDLDLTDLVINAVNDAAVAAPTHRWVKNLPDEPVWVNGDHA 23504

Query:    277 RLHQVMANLLXNARTHTPPGTSVTVALSADAEG----WVTVAVTDDGPGIPPELLPDVFE 332
              RLHQ+++NLL NA  HT PG +VT+ ++    G     V ++VTDDGP I PE+LP +F+
Sbjct: 23503 RLHQLVSNLLTNAWVHTQPGVTVTIGITCHRTGPNAPCVELSVTDDGPDIDPEILPHLFD 23324

Query:    333 RFARXDSSRSPREGSTGLGLAIVAAVVQ 360
              RF R   SRS   G  GLGLAIV+++V+
Sbjct: 23323 RFVRASKSRSNGSGH-GLGLAIVSSIVK 23243
```

CDS complement (23144..24673)
/db_xref="SPTREMBL:P96368"
/note="Rv1032c, (MTCY10G2.17). Probable two component sensor protein.

/gene="Rv1032c"
/product="hypothetical protein Rv1032c"
/translation=

"MIPDRNTRSRKAPCWRPRSLRQQLLLGVLAVVTVVLVAVGVVSVLSLSGYVTAMNDAELVES
LHALNHSYTRYRDSAQTSTPTGNLPMSQAVLEFTGQTPGNLIAVLHDGVVIGSAVFSEDGARPAP
PDVIRAIEAQVWDGGPPRVESLGSLGAYQVDSSAAGADRLFVGVSLSLANQIIARKKVTTVALV
GAALVVTAALTVWVVGYALRPLRRVAATAAEVATMPLTDDDHQISVRVRPGDTDPDNEVGIV
GHTLNRLLDNVDGALAHRVDSDLRMRQFITDASHELRTPLAAIQGYAELTRQDSSDLPPTTEYA
LARIESEARRMTLLVDELLLLSRLSEGEDLETEDLDLTDLVINAVNDAAVAAPTHRWVKNLPDEP
VWVNGDHARLHQLVSNLLTNAWVHTQPGVTVTIGITCHRTGPNAPCVELSVTDDGPDIDPEILP
HLFDRFVRASKSRSNGSGHGLGLAIVSSIVKAHRGSVTAESGNGQTVFRVRLPMIEQQIATTA"

MTCY369

```
  Plus Strand HSPs:

 Score = 465 (163.7 bits), Expect = 2.7e-44, P = 2.7e-44
 Identities = 122/268 (45%), Positives = 160/268 (59%), Frame = +2

Query:    92 RRQFAPLARVSATAREVADLELERGEVRLPTPIVPVDPAVXHTEVGQLGTSXNRMLDRIA 151
             RR   PLA    TA  +   +L+R   R+P       P    TEVG+L  + N ML +I
Sbjct:  2684 RRSLRPLAEFEQTAAAIGAGQLDR---RVPQ----WHP---RTEVGRLSLALNGMLAQIQ 2833

Query:   152 SAL-----SARHA--SETRVRQFVADASHELRTPLAAIRGYTEXAQRKRGELPDDVAHAM 204
              A+      SA  A  SE R+RQF+ DASHELRTPL  IRG+ E  ++        DV   +
Sbjct:  2834 RAVASAESSAEKARDSEDRMRQFITDASHELRTPLTTIRGFAELYRQGAAR---DVGMLL 3004

Query:   205 SRVESETSRMTQLVEDMLLLARLDAGRPLERDRVELSRLVVDTVSDRHVAGPQHKWSLDL 264
             SR+ESE SRM  LV+D+LLLARLDA RPLE  RV+L  L  D   D      P+ + +L++
Sbjct:  3005 SRIESEASRMGLLVDDLLLLARLDAHRPLELCRVDLLALASDAAHDARAMDPKRRITLEV 3184

Query:   265 PED--TVVIDGDEARLHQVMANLLXNARTHTPPGTSVTVALSADAEGWVTVAVTDDGPGI 322
              +   T  + GDE+RL QV+ NL+ NA  HTP    VTV +  + +  + + V DDGPG+
Sbjct:  3185 LDGPGTPEVLGDESRLRQVLRNLVANAIQHTPESADVTVRVGTEGDDAI-LEVADDGPGM 3361

Query:   323 PPELLPDVFERFARXDSSRSPREGSTGLGLAIVAAVV 359
               E    VFERF R DSSR+   G TGLGL+IV ++V
Sbjct:  3362 SQEDALRVFERFYRADSSRARASGGTGLGLSIVDSLV 3472
```

CDS 2144..3601

/db_xref="SPTREMBL:P71815"

/note="Rv0758, (MTCY369.03). *pho*R-like similar to PHOR_BACSU P23545 alkaline phosphatase synthesis sensor.

/gene="*phoR*"

/product="PhoR"

/translation=

"MARHLRGRLPLRVRLVAATLILVATGLVASGIAVTSMLQHRLTSRIDRVLLEEAQIWAQITLPLA
PDPYPGHNPDRPPSRFYVRVISPDGQSYTALNDNTAIPAVPANNDVGRHPTTLPSIGGSKTLWRA
VSVRASDGYLTTVAIDLADVRSTVRSLVLLQVGIGSAVLVVPGVAGYAVVRRSLRPLAEFEQTA
AAIGAGQLDRRVPQWHPRTEVGRLSLALNGMLAQIQRAVASAESSAEKARDSEDRMRQFITDAS
HELRTPLTTIRGFAELYRQGAARDVGMLLSRIESEASRMGLLVDDLLLLARLDAHRPLELCRVDL
LALASDAAHDARAMDPKRRITLEVLDGPGTPEVLGDESRLRQVLRNLVANAIQHTPESADVTVR
VGTEGDDAILEVADDGPGMSQEDALRVFERFYRADSSRARASGGTGLGLSIVDSLVAAHGGAVT
VTTALGEGCCFRVSLPRVSDVDQLSLTPVVPGPP"

Figure 1.7. Blast search of *E. coli gadA* gene (glutamate decarboxylase-alpha) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY77

```
 Minus Strand HSPs::

 Score = 1077 (379.1 bits), Expect = 8.7e-108, Sum P(2) = 8.7e-108
 Identities = 221/435 (50%), Positives = 286/435 (65%), Frame = -1

Query:      31 RFPLHEMRDDVAFQIINDELYLDGNARQNLATFCQTWDDENVHKLMDLSINKNWIDKEEY 90
               R P   M  + A++ I+DEL LDG++R NLATF  TW D    KLM  + +KN IDK+EY
Sbjct: 3851663 RMPDESMDPEAAYRFIHDELMLDGSSRLNLATFVTTWMDPEAEKLMAETFDKNMIDKDEY 3851484

Query:      91 PQSAAIDLRCVNMVADLWHAPAPKNGQ---AVGTNTIGSSEACMLGGMAMKWRWRKRMEA 147
               P +AAI+ RCV+MVADL+HA   ++     A G +TIGSSEA MLGG+A+KWRWR+R+ +
Sbjct: 3851483 PATAAIEARCVSMVADLFHAEGLRDHDPTSATGVSTIGSSEAVMLGGLALKWRWRQRVGS 3851304

Query:     148 -AGKPTDKPNLVCGP-VQICWHKFARYWDVELREIPMRPGQLFMDPKRMIEACDENTIGV 205
                G+    PNLV G  VQ+ W KF RY+DVE R +PM  G+  + P++++ A DENTIGV
Sbjct: 3851303 WKGR---MPNLVMGSNVQVVWEKFCRYFDVEPRYLPMERGRYVITPEQVLAAVDENTIGV 3851133

Query:     206 VPTFGVTYTGNYEFPQPLHDALDKFQADTGIDIDMHIDAASGGFLAPFVAPDIVWDFRLP 265
               V   G TYTG  E    +  ALDK  A  G+D+ +H+DAASGGF+ PF+ PD+VWDFRLP
Sbjct: 3851132 VAILGTTYTGELEPIAEICAALDKLAAGGGVDVPVHVDAASGGFVVPFLHPDLVWDFRLP 3850953

Query:     266 RVKSISASGHKFGLAPLGCGWVIWRDEEALPQELVFNVDYLGGQIGTFAINFSRPAGQVI 325
               RV SI+ SGHK+GL   G G+V+WR  E LP++LVF V+YLGG + TF +NFSRP  QV+
Sbjct: 3850952 RVVSINVSGHKYGLTYPGVGFVVWRGPEHLPEDLVFRVNYLGGDMPTFTLNFSRPGNQVV 3850773

Query:     326 AQYYEFLRLGREGYTKVQNASYQVAAYLADEIAKLGPYEFICTGRPDEGIPAVCFKLKDG 385
                QYY FLRLGR+GYTKV  A    A +L D++ ++   E I  G     IP V F+L
Sbjct: 3850772 GQYYNFLRLGRDGYTKVMQALSHTARWLGDQLREVDHCEVISDG---SAIPVVSFRLAG- 3850605

Query:     386 EDPGYTLYDLSERLRLRGWQVPAFTLGGEATDIVVMRIMCRRGFEMDFAELLLEDYKASL 445
                D GYT +D+S  LR  GWQVPA+T+   ATD+ V+RI+ R G   D A  L +D   +L
Sbjct: 3850604 -DRGYTEFDVSHELRTFGWQVPAYTMPDNATDVAVLRIVVREGLSADLARALHDDAVTAL 3850428

Query:     446 KYLSDHPKLQGIAQQNSFKH 465
                 L D  K  G      F H
Sbjct: 3850427 AAL-DKVKPGGHFDAQHFAH 3850371
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/product="GadB"
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WRQRVGSWKGRMPNLVMGSNVQVVWEKFCRYFDVEPRYLPMERGRYVITPEQVLAAVDENTIGVVAILGT
TYTGELEPIAEICAALDKLAAGGGVDVPVHVDAASGGFVVPFLHPDLVWDFRLPRVVSINVSGHKYGLTYPG
VGFVVWRGPEHLPEDLVFRVNYLGGDMPTFTLNFSRPGNQVVGQYYNFLRLGRDGYTKVMQALSHTARWL
GDQLREVDHCEVISDGSAIPVVSFRLAGDRGYTEFDVSHELRTFGWQVPAYTMPDNATDVAVLRIVVREGLS
ADLARALHDDAVTALAALDKVKPGGHFDAQHFAH"

Figure 1.8. Blast search of *E. coli gadB* gene (glutamate decarboxylase-beta) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

```
MTCY77

  Minus Strand HSPs:

 Score = 1077 (379.1 bits), Expect = 8.7e-108, Sum P(2) = 8.7e-108
 Identities = 221/435 (50%), Positives = 286/435 (65%), Frame = -1

Query:      31 RFPLHEMRDDVAFQIINDELYLDGNARQNLATFCQTWDDENVHKLMDLSINKNWIDKEEY 90
               R P   M  + A++ I+DEL LDG++R NLATF  TW D    KLM  + +KN IDK+EY
Sbjct: 3851663 RMPDESMDPEAAYRFIHDELMLDGSSRLNLATFVTTWMDPEAEKLMAETFDKNMIDKDEY 3851484

Query:      91 PQSAAIDLRCVNMVADLWHAPAPKNGQ---AVGTNTIGSSEACMLGGMAMKWRWRKRMEA 147
               P +AAI+ RCV+MVADL+HA   ++     A G +TIGSSEA MLGG+A+KWRWR+R+ +
Sbjct: 3851483 PATAAIEARCVSMVADLFHAEGLRDHDPTSATGVSTIGSSEAVMLGGLALKWRWRQRVGS 3851304

Query:     148 -AGKPTDKPNLVCGP-VQICWHKFARYWDVELREIPMRPGQLFMDPKRMIEACDENTIGV 205
              G+    PNLV G  VQ+ W KF RY+DVE R +PM  G+  + P++++ A DENTIGV
Sbjct: 3851303 WKGR---MPNLVMGSNVQVVWEKFCRYFDVEPRYLPMERGRYVITPEQVLAAVDENTIGV 3851133

Query:     206 VPTFGVTYTGNYEFPQPLHDALDKFQADTGIDIDMHIDAASGGFLAPFVAPDIVWDFRLP 265
               V   G TYTG  E    +  ALDK  A  G+D+ +H+DAASGGF+ PF+ PD+VWDFRLP
Sbjct: 3851132 VAILGTTYTGELEPIAEICAALDKLAAGGGVDVPVHVDAASGGFVVPFLHPDLVWDFRLP 3850953

Query:     266 RVKSISASGHKFGLAPLGCGWVIWRDEEALPQELVFNVDYLGGQIGTFAINFSRPAGQVI 325
               RV SI+ SGHK+GL   G G+V+WR  E LP++LVF V+YLGG + TF +NFSRP  QV+
Sbjct: 3850952 RVVSINVSGHKYGLTYPGVGFVVWRGPEHLPEDLVFRVNYLGGDMPTFTLNFSRPGNQVV 3850773

Query:     326 AQYYEFLRLGREGYTKVQNASYQVAAYLADEIAKLGPYEFICTGRPDEGIPAVCFKLKDG 385
               QYY FLRLGR+GYTKV  A    A +L D++ ++   E I  G     IP V F+L
Sbjct: 3850772 GQYYNFLRLGRDGYTKVMQALSHTARWLGDQLREVDHCEVISDG---SAIPVVSFRLAG- 3850605

Query:     386 EDPGYTLYDLSERLRLRGWQVPAFTLGGEATDIVVMRIMCRRGFEMDFAELLLEDYKASL 445
               D GYT +D+S  LR  GWQVPA+T+   ATD+ V+RI+ R G   D A  L +D   +L
Sbjct: 3850604 -DRGYTEFDVSHELRTFGWQVPAYTMPDNATDVAVLRIVVREGLSADLARALHDDAVTAL 3850428

Query:     446 KYLSDHPKLQGIAQQNSFKH 465
               L D  K  G      F H
Sbjct: 3850427 AAL-DKVKPGGHFDAQHFAH 3850371
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/db_xref="SPTREMBL:O06249"
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KWRWRQRVGSWKGRMPNLVMGSNVQVVWEKFCRYFDVEPRYLPMERGRYVITPEQVLAAVDENTIGVVAI
LGTTYTGELEPIAEICAALDKLAAGGGVDVPVHVDAASGGFVVPFLHPDLVWDFRLPRVVSINVSGHKYG
LTYPGVGFVVWRGPEHLPEDLVFRVNYLGGDMPTFTLNFSRPGNQVVGQYYNFLRLGRDGYTKVMQALSH
TARWLGDQLREVDHCEVISDGSAIPVVSFRLAGDRGYTEFDVSHELRTFGWQVPAYTMPDNATDVAVLRI
VVREGLSADLARALHDDAVTALAALDKVKPGGHFDAQHFAH
```

Figure 1.9. Blast search of *E. coli icd* gene (isocitrate dehydrogenase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTV012

```
Plus Strand HSPs:

Score = 157 (55.3 bits), Expect = 2.2e-16, Sum P(2) = 2.2e-16
 Identities = 50/139 (35%), Positives = 72/139 (51%), Frame = -1

Query:       12 PENPI-IPYIEGDGIGVDVTPAMLKVVDAAVEKAYKGERKISWMEIYTGEKSTQVYGQDV 70
                P  P+ +  I GDGIG +VT   +KV+DA V     G +K S+ ++  G +     G+
Sbjct: 3353477 PSTPMKLAIIAGDGIGPEVTAEAVKVLDAVVP----GVQKTSY-DL--GARRFHATGEV- 3353322

Query:       71 WLPAETLDLIREYRVAIKGPLTTP-VGGGI--RSLNVALRQELDLYICLRPVRYYQGTPS 127
                 LP   +  +R +   + G +  P V  G+  R L + LR ELD +I LRP R Y G  S
Sbjct: 3353321 -LPDSVVAELRNHDAILLGAIGDPSVPSGVLERGLLLRLRFELDHHINLRPARLYPGVAS 3353145

Query:      128 PVKHPELTDMVIFRENSEDIYAG 150
                P+      D V+ RE +E  Y G
Sbjct: 3353144 PLSGNPGIDFVVVREGTEGPYTG 3353076
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CDS 1..336
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/note="Rv2995c, (MTV012.09). 3-isopropylmalate dehydrogenase.

/gene="*leuB*"
/product="LeuB"
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GNGGAIRVGTPNEVATEVSVNTAFGVRRVVADAFERARRRRKHLTLVHKTNVLTFAGGLWLRT
VDEVGECYPDVEVAYQHVDAATIHMITDPGRFDVIVTDNLFGDIITDLAAAVCGGIGLAASGNID
ATRANPSMFEPVHGSAPDIAGQGIADPTAAIMSVALLLSHLGEHDAAARVDRAVEAHLATRGSE
RLATSDVGERIAAAL"

Figure 1.10. Blast search of *E. coli aceEF* gene (pyruvate dehydrogenase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY427 Plus Strand HSPs:

```
 Score = 2329 (819.8 bits), Expect = 1.1e-241, Sum P(2) = 1.1e-241
 Identities = 461/879 (52%), Positives = 607/879 (69%), Frame = +1

Query:       7 DVDPIETRDWLQAIESVIREEGVERAQYLIDQLLAEARKGGVNVAAGTGISNYINTIPVE 66
               D+DP ET +WL++ +++++  G  RA+YL+ +LL  A +  V + A T  ++Y+NTIP E
Sbjct: 2512555 DIDPEETSEWLESFDTLLQRCGPSRARYLMLRLLERAGEQRVAIPALTS-TDYVNTIPTE 2512731

Query:      67 EQPEYPGNLELERRIRSAIRWNAIMTVLRASKKDLELGGHMASFQSSATIYDVCFNHFFR 126
                +P +PG+ ++ERR R+ IRWNA + V RA +  + +GGH++++ SSA +Y+V FNHFFR
Sbjct: 2512732 LEPWFPGDEDVERRYRAWIRWNAAIMVHRAQRPGVGVGGHISTYASSAALYEVGFNHFFR 2512911

Query:     127 ARNEQDGGDLVYFQGHISPGVYARAFLEGRLTQEQLDNFRQE-VH-GNGLSSYPHPKLMP 184
                ++   GGD V+ QGH SPG+YARAFLEGRLT EQLD FRQE  H G GL SYPHP+LMP
Sbjct: 2512912 GKSHPGGGDQVFIQGHASPGIYARAFLEGRLTAEQLDGFRQEHSHVGGGLPSYPHPRLMP 2513091

Query:     185 EFWQFPTVSMGLGPIGAIYQAKFLKYLEHRGLKDTSKQTVYAFLGDGEMDEPESKGAITI 244
               +FW+FPTVSMGLGP+ AIYQA+F  YL  RG+KDTS Q V+ FLGDGEMDEPES+G   +
Sbjct: 2513092 DFWEFPTVSMGLGPLNAIYQARFNHYLHDRGIKDTSDQHVWCFLGDGEMDEPESRGLAHV 2513271

Query:     245 ATREKLDNLVFVINCNLQRLDGPVTGNGKIINELEGIFEGAGWNVIKVMWGSRWDELLRK 304
                  E LDNL FVINCNLQRLDGPV GNGKII ELE  F GAGWNVIKV+WG  WD LL
Sbjct: 2513272 GALEGLDNLTFVINCNLQRLDGPVRGNGKIIQELESFFRGAGWNVIKVVWGREWDALLHA 2513451

Query:     305 DTSGKLIQLMNETVDGDYQTFKSKDGAYVREHFFGKYPETAALVADWTDEQIWALNRGGH 364
               D  G L+ LMN T DGDYQT+K+ DG YVR+HFFG+ P T ALV + +D+ IW L RGGH
Sbjct: 2513452 DRDGALVNLMNTTPDGDYQTYKANDGGYVRDHFFGRDPRTKALVENMSDQDIWNLKRGGH 2513631

Query:     365 DPKKIYAAFKKAQETKGKATVILAHTIKGYGMGDAAEGKNIAHQVKKMNMDGVRHIRDRF 424
               D +K+YAA++ A + KG+ TVILA TIKGY +G   EG+N  HQ+KK+ ++ ++  RD
Sbjct: 2513632 DYRKVYAAYRAAVDHKGQPTVILAKTIKGYALGKHFEGRNATHQMKKLTLEDLKEFRDTQ 2513811

Query:     425 NVPVSDADIEKLPYIT--FPEG--SEEHTYLHAQRQKLHGYLPSRQPNFTEKLELPSLQD 480
                +PVSDA +E+ PY+   +  G  + E  Y+  +R+ L G++P R+   ++ L LP  +D
Sbjct: 2513812 RIPVSDAQLEENPYLPPYYHPGLNAPEIRYMLDRRRALGGFVPERRTK-SKALTLPG-RD 2513985

Query:     481 FGALLEEQS--KEISTTIAFVRALNVMLKNKSIKDRLVPIIADEARTFGMEGLFRQIGIY 538
                 A L++ S  +E++TT+A VR    +L++K I  R+VPII DEARTFGM+  F  + IY
Sbjct: 2513986 IYAPLKKGSGHQEVATTMATVRTFKEVLRDKQIGPRIVPIIPDEARTFGMDSWFPSLKIY 2514165

Query:     539 SPNGQQYTPQDREQVAYYKEDEKGQILQEGINELGAGCSWLAAATSYSTNNLPMIPFYIY 598
               + NGQ YT  D + +  YKE E GQIL EGINE G+  S++AA TSY+T+N PMIP YI+
Sbjct: 2514166 NRNGQLYTAVDADLMLAYKESEVGQILHEGINEAGSVGSFIAAGTSYATHNEPMIPIYIF 2514345

Query:     599 YSMFGFQRIGDLCWAAGDQQARGFLIGGTSGRTTLNGEGLQHEDGHSHIQSLTIPNCISY 658
               YSMFGFQR GD  WAA DQ ARGF++G T+GRTTL GEGLQH DGHS + + T P  ++Y
Sbjct: 2514346 YSMFGFQRTGDSFWAAADQMARGFVLGATAGRTTLTGEGLQHADGHSLLLAATNPAVVAY 2514525

Query:     659 DPAYAYEVAVIMHDGLERMYGEKQENVYYYITTLNENYHMPAMPEGAE-EGIRKGIYKLE 717
               DPA+AYE+A I+  GL RM GE  EN+++YIT  NE Y  P  PE  + EG+ +GIY+
Sbjct: 2514526 DPAFAYEIAYIVESGLARMCGENPENIFFYITVYNEPYVQPPEPENFDPEGVLRGIYRYH 2514705

Query:     718 TI-EGSKGKVQLLGSGSILRHVREAAEILAKDYGVGSDVYSVTSFTELARDGQDCERWNM 776
                  E    K Q+L SG  +     AA++LA ++ V +DV+SVTS+ EL RDG   E   +
Sbjct: 2514706 AATEQRTNKAQILASGVAMPAALRAAQMLAAEWDVAADVWSVTSWGELNRDGVAIETEKL 2514885

Query:     777 LHPLETPRVPYIAQVMNDA--PAVASTDYMKLFAEQVRTYVPADDYRVLGTDGFGRSDSR 834
                HP     VPY+ + + +A  P +A +D+M+   EQ+R +VP   Y  LGTDGFG SD+R
Sbjct: 2514886 RHPDRPAGVPYVTRALENARGPVIAVSDWMRAVPEQIRPWVPGT-YLTLGTDGFGFSDTR 2515062

Query:     835 ENLRHHFEVDASYVVVAALGELAKRGEIDKKVVADAIAKFNID 877
                  R +F  DA   VVA L  LA  GEID  V   A  ++ ID
Sbjct: 2515063 PAARRYFNTDAESQVVAVLEALAGDGEIDPSVPVAAARQYRID 2515191
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/product="AceE"
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HPGGGDQVFIQGHASPGIYARAFLEGRLTAEQLDGFRQEHSHVGGGLPSYPHPRLMPDFWEFPT
VSMGLGPLNAIYQARFNHYLHDRGIKDTSDQHVWCFLGDGEMDEPESRGLAHVGALEGLDNLT
FVINCNLQRLDGPVRGNGKIIQELESFFRGAGWNVIKVVWGREWDALLHADRDGALVNLMNTT
PDGDYQTYKANDGGYVRDHFFGRDPRTKALVENMSDQDIWNLKRGGHDYRKVYAAYRAAVD
HKGQPTVILAKTIKGYALGKHFEGRNATHQMKKLTLEDLKEFRDTQRIPVSDAQLEENPYLPPY
YHPGLNAPEIRYMLDRRRALGGFVPERRTKSKALTLPGRDIYAPLKKGSGHQEVATTMATVRTF
KEVLRDKQIGPRIVPIIPDEARTFGMDSWFPSLKIYNRNGQLYTAVDADLMLAYKESEVGQILHE
GINEAGSVGSFIAAGTSYATHNEPMIPIYIFYSMFGFQRTGDSFWAAADQMARGFVLGATAGRTT
LTGEGLQHADGHSLLLAATNPAVVAYDPAFAYEIAYIVESGLARMCGENPENIFFYITVYNEPYV
QPPEPENFDPEGVLRGIYRYHAATEQRTNKAQILASGVAMPAALRAAQMLAAEWDVAADVWS
VTSWGELNRDGVAIETEKLRHPDRPAGVPYVTRALENARGPVIAVSDWMRAVPEQIRPWVPGT
YLTLGTDGFGFSDTRPAARRYFNTDAESQVVAVLEALAGDGEIDPSVPVAAARQYRIDDVAAAP
EQTTDPGPGA"

Figure 1.11. Blast search of *E. coli purB* gene (adenylosuccinate lyase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY369

```
  Plus Strand HSPs:

 Score = 166 (58.4 bits), Expect = 1.6e-09, Sum P(2) = 1.6e-09
 Identities = 85/302 (28%), Positives = 135/302 (44%), Frame = +3

Query:     43 WLQKLAAHAAIKEVPAFAADAIGYLDAIVASFSEEDAARIKTIERTTNHDVKAVEYFLKE 102
              WL  L A A   E+    AD++  L        + D A I   ER   HDVKA
Sbjct: 870102 WLAVLRAQA---ELGVAVADSV--LADYERVVDDVDLASISARERVLRHDVKA------- 870245

Query:    103 KVAEIPELHAVSEFIHFACTSEDINNLSHALMLKTARDEVILAYWRQLIDGLKDLAVQYR 162
              ++ E   L A  E +H   TS D+      L ++ +  EVI A+    +  L + AV YR
Sbjct: 870246 RIEEFNAL-AGHEHVHKGMTSRDLTENVEQLQIRRSL-EVIFAHGVAAVARLAERAVSYR 870419

Query:    163 DIPLLSRTHGQPATPSTIGKEMANVAYRME---RQYRQLNQVEILGKINGAVGNYNAHI- 218
              D+ +  R+H   A  +T+GK  A+ A  M    R+ R+L     L  I G +G     +
Sbjct: 870420 DLIMAGRSHNVAAQATTLGKRFASAAQEMMIALRRLRELIDRYPLRGIKGPMGTGQDMLD 870599

Query:    219 ------AAYPEVDWHQFSE--EFVTSLGIQWNPYTTQIEPHDYIAELFDCVARFNTILID 270
                    AA  +++  + ++   F T        Y   ++ HD ++ L    A  +++
Sbjct: 870600 LLGGDRAALADLE-RRVADFLGFATVFNSVGQVYPRSLD-HDVVSALVQLGAGPSSLAHT 870773

Query:    271 FDRDVWGY-IALNHFKQKTIAGEIGSSTMPHKVNPIDFENSEGNLGLSNAVLQHLASKLP 329
               R + G+ +A   F      G++GSS MPHK+N    E   G L +       + ++L
Sbjct: 870774 I-RLMAGHELATEGFAP----GQVGSSAMPHKMNTRSCERVNG-LQVVLRGYASMVAELA 870935

Query:    330 VSRWQR-DLTDSTVLR 344
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ARLAERAVSYRDLIMAGRSHNVAAQATTLGKRFASAAQEMMIALRRLRELIDRYPLRGIKGPMG
TGQDMLDLLGGDRAALADLERRVADFLGFATVFNSVGQVYPRSLDHDVVSALVQLGAGPSSLA
HTIRLMAGHELATEGFAPGQVGSSAMPHKMNTRSCERVNGLQVVLRGYASMVAELAGAQWNE
GDVFCSVVRRVALPDSFFAVDGQIETFLTVLDEFGAYPAVIGRELDRYLPFLATTKVLMAAVRA
GMGRESAHRLISEHAVATALAMREHGAEPDLLDRLAADPRLTLGRDALEAALADKKAFAGAAG
DQVDDVVAMVDALVSRYPDAAKYTPGAIL"

Figure 1.12. Blast search of *E. coli fur* gene (iron Regulator) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY27

```
 Plus Strand HSPs:

 Score = 249 (87.7 bits), Expect = 8.6e-20, Sum P(2) = 8.6e-20
 Identities = 50/132 (37%), Positives = 75/132 (56%), Frame = +1

Query:       8 LKKAGLKVTLPRLKILEVLQEPDNHHVSAEDLYKRLIDMGEEIGLATVYRVLNQFDDAGI 67
               +  AG++ T  R  I  +L+  D+   SA++L+  L   GE IGL TVYR L     +G+
Sbjct: 2641648 MSAAGVRSTRQRAAISTLLETLDDFR-SAQELHDELRRRGENIGLTTVYRTLQSMASSGL 2641824

Query:      68 VTRHNFEGGKSVFELTQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSL 127
               V   + + G+SV+    +HHH HL+C  CG  IE  D  +EA   E+A KHG    +H++
Sbjct: 2641825 VDTLHTDTGESVYRRCSEHHHHHLVCRSCGSTIEVGDHEVEAWAAEVATKHGFSDVSHTI 2642004

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                ++G C+  DCR
Sbjct: 2642005 EIFGTCS--DCR 2642034
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SHTIEIFGTCSDCRS"

CDS 1..150
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/gene="*furA*"
/product="FurA"
/translation=

"MSSVSSIPDYAEQLRTADLRVTRPRVAVLEAVNAHPHADTETIFGAVRFALPDVSRQA
VYDVLHALTAAGLVRKIQPSGSVARYESRVGDNHHHIVCRSCGVIADVDCAVGEAPCL
TASDHNGFLLDEAE VIYWGLCPDC SISDTSRSHP"

Figure 1.13. Blast search of *E. coli gpi* gene (glucose-6-phosphate isomerase) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY10D7

```
 Minus Strand HSPs:

Score = 1376 (484.4 bits), Expect = 7.2e-139, Sum P(3) = 7.2e-139
Identities = 283/543 (52%), Positives = 370/543 (68%), Frame = -1

Query:       7 TQTAAWQALQKHFDEMKDVTIADLFAKDGDRFSKFSATFDDQMLVDYSKNRITEETLAKL 66
               T T AW AL++H D++ +  +   FA D  R  + + +  D + +DYSK+R+T ETLA L
Sbjct: 1056656 TATPAWDALRRHHDQIGNTHLRQFFADDPGRGRELTVSVGD-LYIDYSKHRVTRETLALL 1056480

Query:      67 QDLAKECDLAGAIKSMFSGEKINRTENRAVLHVALRNRSNTPILVDGKDVMPEVNAVLEK 126
                DLA+   L      MF+G  IN +E+RAVLH ALR   +  ++VDG+DV+ +V+AVL+
Sbjct: 1056479 IDLARTAHLEERRDQMFAGVHINTSEDRAVLHTALRLPRDAELVVDGQDVVTDVHAVLDA 1056300

Query:     127 MKTFSEAIISGEWKGYTGKAITDVVNIGIGGSDLGPYMVTEALRPYKNH-LNMHFVSNVD 185
               M  F++ + SGEW G TGK I+ VVNIGIGGSDLGP MV +ALR Y +  ++  FVSNVD
Sbjct: 1056299 MGAFTDRLRSGEWTGATGKRISTVVNIGIGGSDLGPVMVYQALRHYADAGISARFVSNVD 1056120

Query:     186 GTHIAEVLKKVNPETTLFLVASKTFTTQETMTNAHSARDWFLKAAGDEKHVAKHFAALST 245
                  +   L  ++P TTLF+VASKTF+T ET+TNA +AR W   A GD   V++HF A+ST
Sbjct: 1056119 PADLIATLADLDPATTLFIVASKTFSTLETLTNATAARRWLTDALGDAA-VSRHFVAVST 1055943

Query:     246 NAKAVGEFGIDTANMFEFWDWVGGRYSLWSAIGLSIVLSIGFDNFVELLSGAHAMDKHFS 305
               N + V +FGI+T NMF FWDWVGGRYS+ SAIGLS++  IG D F + L+G H +D+HF+
Sbjct: 1055942 NKRLVDDFGINTDNMFGFWDWVGGRYSVDSAIGLSLMTVIGRDAFADFLAGFHIIDRHFA 1055763

Query:     306 TTPAEKNLPVLVALIGIWYNNFFGAETEAILPYDQYMHRFAAYFQQGNMESNGKYVDRNG 365
               T P E N PVL+ LIG+WY+NFFGA++  +LPY   + RF AY QQ  MESNGK    +G
Sbjct: 1055762 TAPLESNAPVLLGLIGLWYSNFFGAQSRTVLPYSNDLSRFPAYLQQLTMESNGKSTRADG 1055583

Query:     366 NVVDYQTGPIIWGEPGTNGQHAFYQLIHQGTKMVPCDFI--APAITHNPLSDH----HQK 419
               + V   TG I WGEPGTNGQHAFYQL+HQGT++VP DFI  A  +   P ++     H
Sbjct: 1055582 SPVSADTGEIFWGEPGTNGQHAFYQLLHQGTRLVPADFIGFAQPLDDLPTAEGTGSMHDL 1055403

Query:     420 LLSNFFAQTEALAFGKSREVVEQEYRDQGKDPATLDYVVPFKVFEGNRPTNSILLREITP 479
               L+SNFFAQT+ LAFGK+ E +  +    G  PA   +VV  KV  GNRP+ SIL   +TP
Sbjct: 1055402 LMSNFFAQTQVLAFGKTAEEIAAD----GT-PA---HVVAHKVMPGNRPSTSILASRLTP 1055247

Query:     480 FSLGALIALYEHKIFTQGVILNIFTFDQWGVELGKQLANRILPELKD-DKEISSHDSSTN 538
                 LG LIALYEH++FT+GV+  I +FDQWGVELGK  A  +LP +          DSST+
Sbjct: 1055246 SVLGQLIALYEHQVFTEGVVWGIDSFDQWGVELGKTQAKALLPVITGAGSPPPQSDSSTD 1055067

Query:     539 GLINRYKAWRG 549
               GL+ RY+  RG
Sbjct: 1055066 GLVRRYRTERG 1055034
```

CDS complement(4572..6233)
/note="Rv0946c, (MTCY10D7.28).Probable glucose-6-phosphate isomerase.

/gene="*pgi*"
/product="Pgi"
/translation=

"MTSAPIPDITATPAWDALRRHHDQIGNTHLRQFFADDPGRGRELTVSVGDLYIDYSKHRV
TRETLALLIDLARTAHLEERRDQMFAGVHINTSEDRAVLHTALRLPRDAELVVDGQDVVT
DVHAVLDAMGAFTDRLRSGEWTGATGKRISTVVNIGIGGSDLGPVMVYQALRHYADAGI
SARFVSNVDPADLIATLADLDPATTLFIVASKTFSTLETLTNATAARRWLTDALGDAAVSR
HFVAVSTNKRLVDDFGINTDNMFGFWDWVGGRYSVDSAIGLSLMTVIGRDAFADFLAGF
HIIDRHFATAPLESNAPVLLGLIGLWYSNFFGAQSRTVLPYSNDLSRFPAYLQQLTMESNG
KSTRADGSPVSADTGEIFWGEPGTNGQHAFYQLLHQGTRLVPADFIGFAQPLDDLPTAEG
TGSMHDLLMSNFFAQTQVLAFGKTAEEIAADGTPAHVVAHKVMPGNRPSTSILASRLTPS
VLGQLIALYEHQVFTEGVVWGIDSFDQWGVELGKTQAKALLPVITGAGSPPPQSDSSTDG
LVRRYRTERGRAG"

Figure 1.14. Blast search of *E. coli osmY* gene (unknown) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCI237

```
  Plus Strand HSPs:

 Score = 274 (96.5 bits), Expect = 4.9e-24, P = 4.9e-24
 Identities = 79/251 (31%), Positives = 129/251 (51%), Frame = +2

Query:       6 IDTHCHFDF-PPFSGDEEASL-QRAAQAGVGKIIVPATEAENFARVLALAENYQPLYAAL 63
               +D H H D        D    SL +RAA AGV  ++  A + E+   V   AE  + +YAA+
Sbjct: 1127090 VDAHTHLDACGARDADTVRSLVERAAAAGVTAVVTVADDLESARWVTRAAEWDRRVYAAV 1127269

Query:      64 GLHPGMLEKHSDVSLEQLQQALERRPAKVVAVGEIGLDLF--G--D---DPQFERQQ--W 114
                LHP   +  +D +  +L++ L   P +VVAVGE G+D++  G  D   +P  +R+   W
Sbjct: 1127270 ALHPTRADALTDAARAELER-LVAHP-RVVAVGETGIDMYWPGRLDGCAEPHVQREAFAW 1127443

Query:     115 LLDEQLKLAKRYDLPVILHSRRTHDKLAMHLKRHDLPRTGVVHGFSGSLQQAERFVQLGY 174
               +D    LAKR   P+++H+R+    +   L+    P T ++H FS     A   V  G+
Sbjct: 1127444 HID----LAKRTGKPLMIHNRQADRDVLDVLRAEGAPDTVILHCFSSDAAMARTCVDAGW 1127611

Query:     175 KIGVGGTITYPRASKTRDVIAKLPLASLLLETDAPDMPLNGFQGQPNRPEQAARVFAVLC 234
               + + GT+++  A + R+ +  +P+  LL+ETDAP +  +  +G  N P         L
Sbjct: 1127612 LLSLSGTVSFRTARELREAVPLMPVEQLLVETDAPYLTPHPHRGLANEPYCLPYTVRALA 1127791

Query:     235 ELRREPADEIAQALLNN 251
               EL     +E+A    +N
Sbjct: 1127792 ELVNRRPEEVALITTSN 1127842
```

CDS 1..264
/db_xref="SPTREMBL:O08343"
/note="Rv1008, (MTCI237.25). Hypothetical 29.8 kd protein.

/gene="Rv1008"
/product="hypothetical protein Rv1008"
/translation=

"MVDAHTHLDACGARDADTVRSLVERAAAAGVTAVVTVADDLESARWVTRAAEWDRRVYAA
VALHPTRADALTDAARAELERLVAHPRVVAVGETGIDMYWPGRLDGCAEPHVQREAFAWHID
LAKRTGKPLMIHNRQADRDVLDVLRAEGAPDTVILHCFSSDAAMARTCVDAGWLLSLSGTVSF
RTARELREAVPLMPVEQLLVETDAPYLTPHPHRGLANEPYCLPYTVRALAELVNRRPEEVALITT
SNARRAYGLGWMRQ"

Figure 1.15. Blast search of *E. coli fabF* gene (fatty acid synthesis) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY427

```
  Plus Strand HSPs:

 Score = 710 (249.9 bits), Expect = 1.5e-68, Sum P(2) = 1.5e-68
 Identities = 165/405 (40%), Positives = 225/405 (55%), Frame = +3

Query:       5 VVVTGLGMLSPVGNTVESTWKALLAGQSGI-SLIDHFDTS-AYATKFAGLVKDFNCEDII 62
               VVVT +   + +   +ESTWK LLAG+SGI +L D F T    A K  G +KD   +  +
Sbjct: 2518152 VVVTAVTATTSISPDIESTWKGLLAGESGIHALEDEFVTKWDLAVKIGGHLKD-PVDSHM 2518328

Query:      63 SRKEQRKMDAFIQYGIVAGVQAMQDSGLEITEENATRXXXXXXXXXXXXXXXEENHTSLM 122
               R + R+M    + G + G Q  + +G    E +  R                E++  LM
Sbjct: 2518329 GRLDMRRMSYVQRMGKLLGGQLWESAGSP--EVDPDRFAVVVGTGLGGAERIVESY-DLM 2518499

Query:     123 N-GGPRKISPFFVPSTIVNMVAGHLTIMYGLRGPSISIATACTSGVHNIGHAARIIAYGD 181
               N GGPRK+SP  V   + N  A  + +  G R   ++  +AC+SG   I HA R I  GD
Sbjct: 2518500 NAGGPRKVSPLAVQMIMPNGAAAVIGLQLGARAGVMTPVSACSSGSEAIAHAWRQIVMGD 2518679

Query:     182 ADVMVAGGAEKASTPLGVGGFGAARALSTRNDNPQAASRPWDKERDGFVLGDGAGMLVLE 241
               ADV V GG E     L +  F   RA+STRND P+ ASRP+DK+RDGFV G+   ++++E
Sbjct: 2518680 ADVAVCGGVEGPIEALPIAAFSMMRAMSTRNDEPERASRPFDKDRDGFVFGEAGALMLIE 2518859

Query:     242 EYEHAKKRGAKIYAELVGFGMSSDAYHMTSPPEXXXXXXXXXXXXXRDAGIEASQIGYVN 301
               EHAK RGAK  A L+G G++SDA+HM +P                 AG+  + I +VN
Sbjct: 2518860 TEEHAKARGAKPLARLLGAGITSDAFHMVAPAADGVRAGRAMTRSLELAGLSPADIDHVN 2519039

Query:     302 AHGTSTPAGDKAEAQAVKTIFGEAASRVLVSSTKSMTGHLLGAAGAVESIYSILALRDQA 361
               AHGT+TP GD AEA A++ + G    +  V + KS  GH +GA GA+ES+ ++L LRD
Sbjct: 2519040 AHGTATPIGDAAEANAIR-VAG--CDQAAVYAPKSALGHSIGAVGALESVLTVLTLRDGV 2519210

Query:     362 VPPTINLDNPDEGCDLDFVPHEARQVSGMEYTLCNSFGFGGTNGSLIF 409
               +PPT+N + PD   DLD V  E R      Y + NSFGFGG N +L F
Sbjct: 2519211 IPPTLNYETPDPEIDLDVVAGEPRY-GDYRYAVNNSFGFGGHNVALAF 2519351
```

CDS 1..416
/db_xref="SWISS-PROT:Q10524"
/note="Rv2245, (MTCY427.26). Beta-ketoacyl-ACP synthase, involved in meromycolate extension.

/gene="*kasA*"
/product="KasA"
/translation=

"MSQPSTANGGFPSVVVTAVTATTSISPDIESTWKGLLAGESGIHALEDEFVTKWDLAVKIGGHL
KDPVDSHMGRLDMRRMSYVQRMGKLLGGQLWESAGSPEVDPDRFAVVVGTGLGGAERIVESY
DLMNAGGPRKVSPLAVQMIMPNGAAAVIGLQLGARAGVMTPVSACSSGSEAIAHAWRQIVMG
DADVAVCGGVEGPIEALPIAAFSMMRAMSTRNDEPERASRPFDKDRDGFVFGEAGALMLIETEE
HAKARGAKPLARLLGAGITSDAFHMVAPAADGVRAGRAMTRSLELAGLSPADIDHVNAHGTAT
PIGDAAEANAIRVAGCDQAAVYAPKSALGHSIGAVGALESVLTVLTLRDGVIPPTLNYETPDPEID
LDVVAGEPRYGDYRYAVNNSFGFGGHNVALAFGRY"

MTCY427

```
 Score = 594 (209.1 bits), Expect = 2.4e-56, Sum P(2) = 2.4e-56
 Identities = 143/405 (35%), Positives = 211/405 (52%), Frame = +3

Query:       5 VVVTGLGMLSPVGNTVESTWKALLAGQSGI-SLIDHF-DTSAYATKFAG-LVKDFNCEDI 61
               VVVTG+ M + +    E+TWK LL  QSGI +L D F +      +  G L+++F+ +
Sbjct: 2519493 VVVTGIAMTTALATDAETTWKLLLDRQSGIRTLDDPFVEEFDLPVRIGGHLLEEFDHQ-- 2519666

Query:      62 ISRKEQRKMDAFIQYGIVAGVQAMQDSGLEITEENATRXXXXXXXXXXXXXXXEENHTSL 121
               ++R E R+M    +   V   +  +++G    + N  R                ++  +
Sbjct: 2519667 LTRIELRRMGYLQRMSTVLSRRLWENAGSPEVDTN--RLMVSIGTGLGSAEELVFSYDDM 2519840

Query:     122 MNGGPRKISPFFVPSTIVNMVAGHLTIMYGLRGPSISIATACTSGVHNIGHAARIIAYGD 181
                G + +SP  V   + N  A  + +    +   ++  +AC SG   I  A + I  G+
Sbjct: 2519841 RARGMKAVSPLTVQKYMPNGAAAAVGLERHAKAGVMTPVSACASGAEAIARAWQQIVLGE 2520020

Query:     182 ADVMVAGGAEKASTPLGVGGFGAAR-ALSTRNDNPQAASRPWDKERDGFVLGDGAGMLVL 240
               AD  + GG E     + + GF   R  +ST ND+P  A RP+D++RDGFV G+G  +L++
Sbjct: 2520021 ADAAICGGVETRIEAVPIAGFAQMRIVMSTNNDDPAGACRPFDRDRDGFVFGEGGALLLI 2520200

Query:     241 EEYEHAKKRGAKIYAELVGFGMSSDAYHMTSPPEXXXXXXXXXXXXXRDAGIEASQIGYV 300
               E  EHAK RGA I A ++G  ++SD +HM +P               + AG+    I +V
Sbjct: 2520201 ETEEHAKARGANILARIMGASITSDGFHMVAPDPNGERAGHAITRAIQLAGLAPGDIDHV 2520380

Query:     301 NAHGTSTPAGDKAEAQAVKTIFGEAASRVLVSSTKSMTGHLLGAAGAVESIYSILALRDQ 360
               NAH T T  GD AE +A+    G   +R  V + KS  GH +GA GAVESI ++LALRDQ
Sbjct: 2520381 NAHATGTQVGDLAEGRAINNALG--GNRPAVYAPKSALGHSVGAVGAVESILTVLALRDQ 2520554

Query:     361 AVPPTINLDNPDEGCDLDFVPHEARQVSGMEYTLCNSFGFGGTNGSLIF 409
                +PPT+NL N D   DLD V  E R      Y + NSFGFGG N ++ F
Sbjct: 2520555 VIPPTLNLVNLDPEIDLDVVAGEPRP-GNYRYAINNSFGFGGHNVAIAF 2520698
```

CDS 1..438

/db_xref="SWISS-PROT:Q10525"

/note="Rv2246, (MTCY427.27). Beta-ketoacyl-ACP synthase, involved in meromycolate extension.

/gene="*kasB*"

/product="KasB"

/translation=

"MGVPPLAGASRTDMEGTFARPMTELVTGKAFPYVVVTGIAMTTALATDAETTWKLLLDRQSGI
RTLDDPFVEEFDLPVRIGGHLLEEFDHQLTRIELRRMGYLQRMSTVLSRRLWENAGSPEVDTNRL
MVSIGTGLGSAEELVFSYDDMRARGMKAVSPLTVQKYMPNGAAAAVGLERHAKAGVMTPVSA
CASGAEAIARAWQQIVLGEADAAICGGVETRIEAVPIAGFAQMRIVMSTNNDDPAGACRPFDRD
RDGFVFGEGGALLLIETEEHAKARGANILARIMGASITSDGFHMVAPDPNGERAGHAITRAIQLA
GLAPGDIDHVNAHATGTQVGDLAEGRAINNALGGNRPAVYAPKSALGHSVGAVGAVESILTVL
ALRDQVIPPTLNLVNLDPEIDLDVVAGEPRPGNYRYAINNSFGFGGHNVAIAFGRY"

Figure 1.16. Blast search of *E. coli ada* gene (DNA repair) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY130

Minus Strand HSPs:

```
 Score = 233 (82.0 bits), Expect = 2.4e-33, Sum P(2) = 2.4e-33
 Identities = 62/175 (35%), Positives = 89/175 (50%), Frame = -2

Query:   174 GMTAKQFRHGGENLAVRYALADCELGRCLVAESERGICAILLGDDDATLISELQQMF-PA 232
             G + K     GE+  + Y   D  +G   +A    G  ++L      T +  L+Q + P+
Sbjct:  4271 GTSGKPMATAGEDRMIHYRTIDSPIGPLTLA----GHGSVL------TNLRMLEQTYEPS 4122

Query:   233 ADN-APADLMFQQHVREVIASLNQRDTPLTLPLDIRGTAFQQQVWQALRTIPCGETVSYQ 291
               +  P    F   V ++ A      T   + LD+RGT FQQ+VW+AL TIP GET SY
Sbjct:  4121 RTHWTPDPGAFSGAVDQLNAYFAGELTEFDVELDLRGTDFQQRVWKALLTIPYGETRSYG 3942

Query:   292 QLANAIGKPKXXXXXXXXXXXXNKLAIIIPCHRVVRGDGTLSGYRWGVSRKAQLLRRE 348
             ++A+ IG P            N +AII+PCHRV+   G L+GY  G++RK  LL  E
Sbjct:  3941 EIADQIGAPGAARAVGLANGHNPIAIIVPCHRVIGASGKLTGYGGGINRKRALLELE 3771


 Score = 191 (67.2 bits), Expect = 2.4e-33, Sum P(2) = 2.4e-33
 Identities = 46/126 (36%), Positives = 65/126 (51%), Frame = -3


Query:     9 DDQR-WQSVLARDPNADGEFVFAVRTTGIFCRPSCRARHALRENVSFYANASEALAAGFR 67
             D +R ++++ ++D   DG FV AV TTG++CRPSC  R     NV F   A+ A   GFR
Sbjct:  5707 DFERCYRAIQSKDARFDGWFVVAVLTTGVYCRPSCPVRPPFARNVRFLPTAAAAQGEGFR 5528

Query:    68 PCKRCQPEKANAQQH---RLDKITHACRLLEQETPVT--LEALADQVAMSPFHLHRLFKA 122
              CKRC+P+ +        R D +  A RL+   T     +  LA Q+  +   L RL +A
Sbjct:  5527 ACKRCRPDASPGSPEWNVRSDVVARAMRLIADGTVDRDGVSGLAAQLGYTIRQLERLLQA 5348

Query:   123 TTGMTP 128
               G  P
Sbjct:  5347 VVGAGP 5330
```

CDS 1..165
/db_xref="SWISS-PROT:Q10627"
/note="Rv1316c, (MTCY130.01c). Probable methylated-dna--protein-cysteine methytransferase

/gene="*ogt*"
/product="Ogt"
/translation=

"MIHYRTIDSPIGPLTLAGHGSVLTNLRMLEQTYEPSRTHWTPDPGAFSGAVDQLNAYFAGELTE
FDVELDLRGTDFQQRVWKALLTIPYGETRSYGEIADQIGAPGAARAVGLANGHNPIAIIVPCHRVI
GASGKLTGYGGGINRKRALLELEKSRAPADLTLFD"

Figure 1.17. Blast search of *E. coli rpoS* gene (sigma factor) against the *M. tuberculosis* H37Rv database.

Sequence similarities between the two sequences are shown and the cosmids containing these genes identified. The protein sequences of the genes of *M. tuberculosis* are shown.

MTCY05A6

```
  Plus Strand HSPs:

 Score = 503 (177.1 bits), Expect = 6.1e-47, Sum P(3) = 6.1e-47
 Identities = 101/231 (43%), Positives = 152/231 (65%), Frame = +3

Query:       84 LRGDVASRRRMIESNLRLVVKIARRYGNRGLALLDLIEEGNLGLIRAVEKFDPERGFRFS 143
                +R   A+RR ++E+NLRLVV +A+RY  RG+ LLDLI+EGNLGLIRA+EKFD  +GF+FS
Sbjct: 3022695 VRDGEAARRHLLEANLRLVVSLAKRYTGRGMPLLDLIQEGNLGLIRAMEKFDYTKGFKFS 3022874

Query:      144 TYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYLRTARELSHKLDHEPSAEEIAEQLD 203
                TYATWWIRQ I R + +Q+RTIRLP+H+V+++N   R  RE+   L  E + EE+A +
Sbjct: 3022875 TYATWWIRQAITRGMADQSRTIRLPVHLVEQVNKLARIKREMHQHLGREATDEELAAESG 3023054

Query:      204 KPVDDVSRMLRLNERITSVDTPLGGDSEKALLDILADEKENGPEDTTQDDDMKQSIVKWL 263
                 P+D ++ +L  +    S+D P+G + E  L D + D +    E+    + +   I   L
Sbjct: 3023055 IPIDKINDLLEHSRDPVSLDMPVGSEEEAPLGDFIEDAEAMSAENAVIAELLHTDIRSVL 3023234

Query:      264 FELNAKQREVLARRFGLLGYEAATLEDVGREIGLTRERVRQIQVEGLRRLR 314
                  L+ ++ +V+  RFGL   +  TL+ +G+  GL+RERVRQI+ + + +LR
Sbjct: 3023235 ATLDEREHQVIRLRFGLDDGQPRTLDQIGKLFGLSRERVRQIERDVMSKLR 3023387
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CDS 25826..26797
/db_xref="SPTREMBL:Q59563"
/note="MTCY05A6.31. RNA polymerase sigma factor.

/gene="*sigB*"
/product="SigB"
/translation=

"MADAPTRATTSRVDSDLDAQSPAADLVRVYLNGIGKTALLNAAGEVELAKRIEAGLYAEHLLETRKRLGE
NRKRDLAAVVRDGEAARRHLLEANLRLVVSLAKRYTGRGMPLLDLIQEGNLGLIRAMEKFDYTKGFKFST
YATWWIRQAITRGMADQSRTIRLPVHLVEQVNKLARIKREMHQHLGREATDEELAAESGIPIDKINDLLEHS
RDPVSLDMPVGSEEEAPLGDFIEDAEAMSAENAVIAELLHTDIRSVLATLDEREHQVIRLRFGLDDGQPRTLD
QIGKLFGLSRERVRQIERDVMSKLRHGERADRLRSYAS"

MTCY05A6

```
Plus Strand HSPs:

 Score = 486 (171.1 bits), Expect = 5.4e-45, Sum P(2) = 5.4e-45
 Identities = 113/282 (40%), Positives = 170/282 (60%), Frame = +3

Query:       41 AEEELLSQGATQRVLDATQLY--LGEIGYS-PLLTAEEEVYFARRALRGDVASRRRMIES 97
                AEEE+      +  L ATQL   L E G   P     + ++  R    GD A +  ++E+
Sbjct: 3018558 AEEEVELAKRIEAGLYATQLMTELSERGEKLPAAQRRDMMWICRD---GDRA-KNHLLEA 3018725

Query:       98 NLRLVVKIARRYGNRGLALLDLIEEGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERA 157
                NLRLVV +A+RY  RG+A LDLI+EGNLGLIRAVEKFD  +G++FSTYATWWIRQ I RA
Sbjct: 3018726 NLRLVVSLAKRYTGRGMAFLDLIQEGNLGLIRAVEKFDYTKGYKFSTYATWWIRQAITRA 3018905

Query:      158 IMNQTRTIRLPIHIVKELNVYLRTARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNE 217
                + +Q RTIR+P+H+V+ +N   R  REL   L  EP+ EE+A+++D   + V  + +
Sbjct: 3018906 MADQARTIRIPVHMVEVINKLGRIQRELLQDLGREPTPEELAKEMDITPEKVLEIQQYAR 3019085

Query:      218 RITSVDTPLGGDSEKALLDILADEKENGPEDTTQDDDMKQSIVKWLFELNAKQREVLARR 277
                   S+D  +G + +  L D + D +     D      ++  +   L  L+ ++  V+  R
Sbjct: 3019086 EPISLDQTIGDEGDSQLGDFIEDSEAVVAVDAVSFTLLQDQLQSVLDTLSEREAGVVRLR 3019265

Query:      278 FGLLGYEAATLEDVGREIGLTRERVRQIQVEGLRRLREILQTQGL 322
                FGL   +  TL+++G+  G+TRER+RQI+ + + +LR   ++Q L
Sbjct: 3019266 FGLTDGQPRTLDEIGQVYGVTRERIRQIESKTMSKLRHPSRSQVL 3019400
```

CDS 21832..23418

/db_xref="SWISS-PROT:Q60162"

/note="Rv2703, (MTCY05A6.24). RNA polymerase sigma factor.

/gene="*sigA*"

/product="SigA"

/translation=

"MAATKASTATDEPVKRTATKSPAASASGAKTGAKRTAAKSASGSPPAKRATKPAARSVKPASAPQDTTTS
TIPKRKTRAAAKSAAAKAPSARGHATKPRAPKDAQHEAATDPEDALDSVEELDAEPDLDVEPGEDLDLDA
ADLNLDDLEDDVAPDADDDLDSGDDEDHEDLEAEAAVAPGQTADDDEEIAEPTEKDKASGDFVWDEDES
EALRQARKDAELTASADSVRAYLKQIGKVALLNAEEEVELAKRIEAGLYATQLMTELSERGEKLPAAQRR
DMMWICRDGDRAKNHLLEANLRLVVSLAKRYTGRGMAFLDLIQEGNLGLIRAVEKFDYTKGYKFSTYAT
WWIRQAITRAMADQARTIRIPVHMVEVINKLGRIQRELLQDLGREPTPEELAKEMDITPEKVLEIQQYAREPI
SLDQTIGDEGDSQLGDFIEDSEAVVAVDAVSFTLLQDQLQSVLDTLSEREAGVVRLRFGLTDGQPRTLDEIG
QVYGVTRERIRQIESKTMSKLRHPSRSQVLRDYLD"

APPENDIX 2.

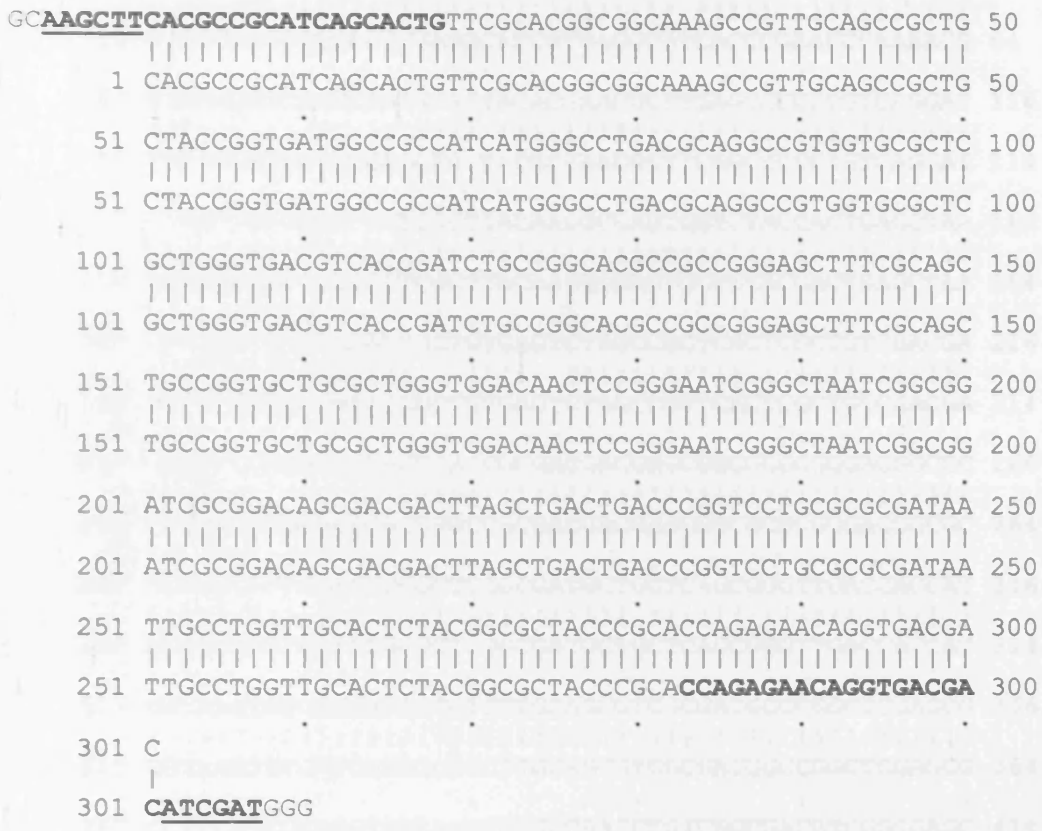

Figure 2.1. Sequence of P*pd* PCR product by automated sequencing.

The 301 bp P*pd* PCR amplified DNA fragment is shown sequenced using primers MTCY159-F and MTCY159-R. The forward and reverse primers are shown in bold and the restriction sites *Cla* I and *Hind* III incorporated in the primer sequence are shown in bold and underlined. This forward and reverse sequence have been matched using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

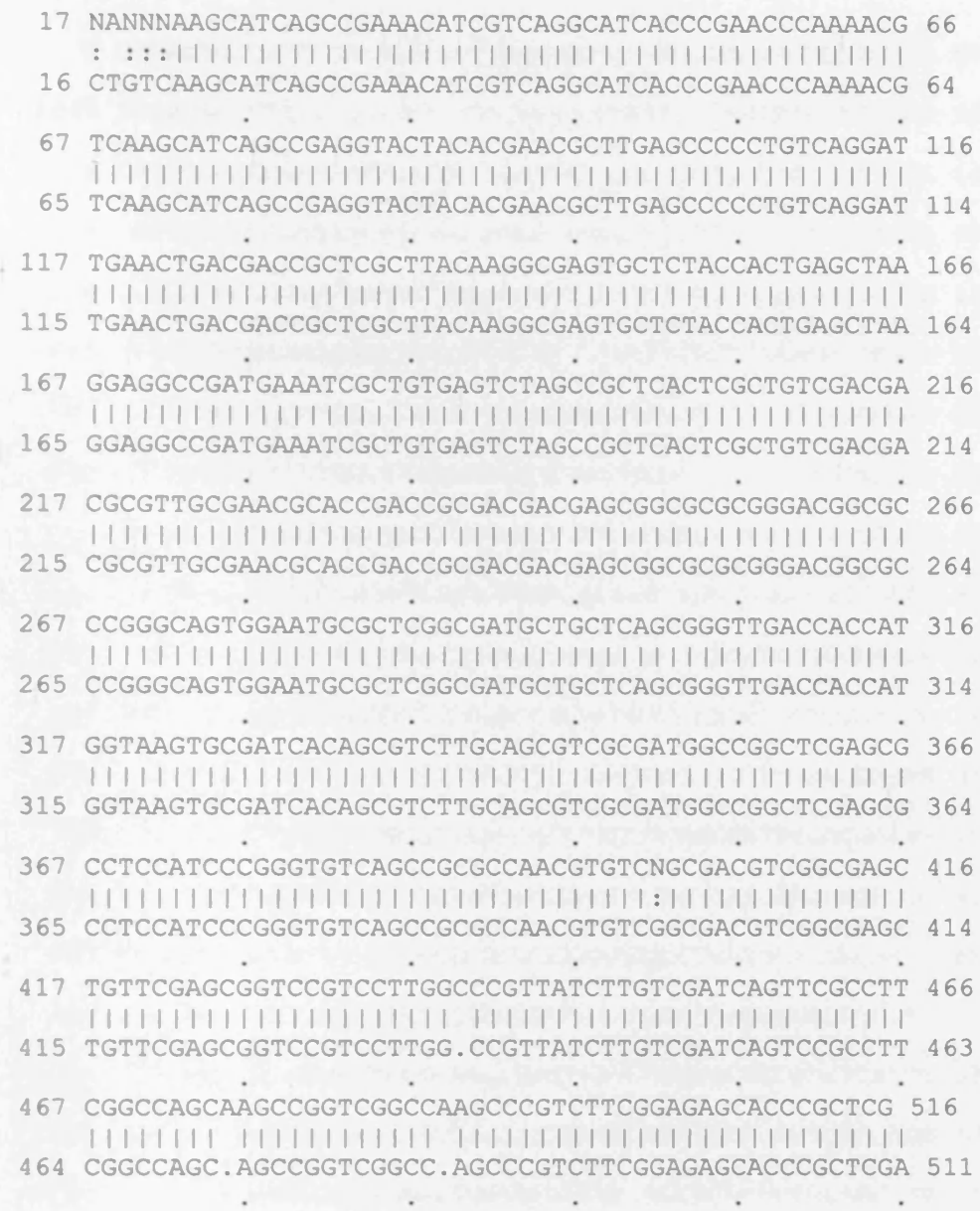

Figure 2.2. Sequence of PCR product P*phoPR* by automated sequencing.

Part of the forward sequence of 1065 bp P*phoPR* PCR amplified DNA fragment is shown sequenced using primer MTCY159-F. The forward sequence of the 1065 bp P*phoPR* amplified DNA fragment (top strand) is shown aligned against the 5' region upstream of the gene *phoPR* of *M. tuberculosis* (bottom strand). This sequence has been matched to the original sequence (Figure 4.10) using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

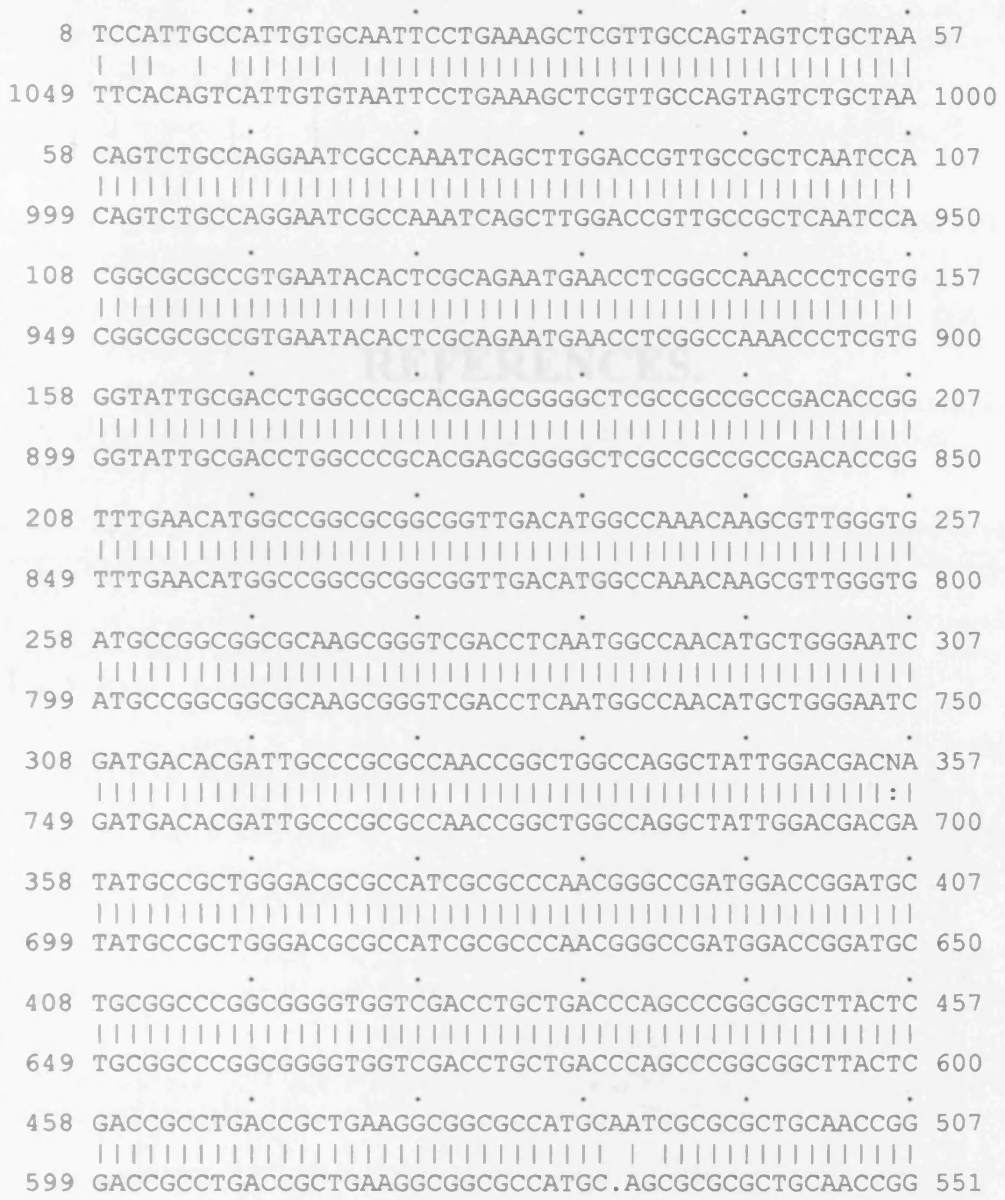

Figure 2.3. Sequence of PCR product P*phoPR* by automated sequencing.

Part of the reverse sequence of 1065 bp P*phoPR* PCR amplified DNA fragment is shown sequenced using primer MTCY159-R. The reverse sequence of the 1065 bp P*phoPR* amplified DNA fragment (top strand) is shown aligned against the 5' region upstream of the gene *phoPR* of *M. tuberculosis* (bottom strand). This sequence has been matched to the original sequence (Figure 4.10) using the 'BESTFIT' program from the University of Wisconsin Genetics Computer Group (GCG Version 9.1) suite of programs (Genetics Computer Group, 1991).

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