"Identification and characterisation of endogenous inducible promoters in *Mycobacterium tuberculosis*"

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Declaration

I declare that this thesis is my original work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Dorothée Laura Schuessler

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Thank you.

This thesis is dedicated to my grandmother Magdalene, who suffered from tuberculosis.

Abstract

Mycobacterium tuberculosis is one of the world's most devastating pathogens. Despite completion of the genome sequence in 1998, research progress has been hampered by a lack of genetic tools and the difficulty of working with the organism. Existing genetic systems are limited by their lack of tight regulation or genetic instability. The aim of this study was to characterise and utilise a range of promoters to express mycobacterial genes in a controllable fashion by generating knockdown strains of a number of target genes, using both sense and antisense approaches. This would help to elucidate the function of a particular gene of interest and identify or validate new drug targets. Sets of genes shown to be inducible by certain stimuli such as tetracycline (Rv0277c, Rv0608, Rv0748, Rv1015c, Rv2487c and Rv3898c), streptomycin (*whiB7*), sodium dodecyl sulphate or ethanol (*whiB6*), hypoxia, nitric oxide and stationary phase (Rv2625c, Rv2626c, Rv2627c and *hspX*), or salicylate (Rv0560c) were selected from the literature. The upstream region of each gene was cloned in front of a reporter gene and activity was tested in *M. smegmatis* and/or *M. tuberculosis.* No inducible promoter activity was found for the upstream regions of the tetracycline, streptomycin, sodium dodecyl sulphate or ethanol-responsive genes. Inducible promoter activity was found for some of the hypoxia-responsive genes and was monitored in relation to growth phase in *M. tuberculosis* wild type, a *dosR* deletion mutant and a Rv2625c deletion mutant. The upstream region of Rv0560c was found to contain a salicylate-inducible promoter. Promoter elements of this promoter were identified and characterised in *M. tuberculosis*. Attempts to use the most promising promoters in an antisense setting using the reporter gene *lacZ* or the mycobacterial gene *rpoB* were unsuccessful.

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List of abbreviations and units

°C	Degree Celsius
2CR	Two-component regulatory system
2D	Two dimensional
A	Adenine
ACDP	Advisory Committee on Dangerous Pathogens
ASA	Acetyl salicylic acid
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guérin
bp	Base pair
BLAST	Basic Local Alignment Search Tool
C	Cytosine
CBS	Cystathionine-ß-synthase
cDNA	Complementary DNA
CHP	Conserved hypothetical protein
CPRG	Chlorophenol red ß-D-galactopyranoside
d	Day
dATPS	Deoxyadenosine triphosphates
DeADMAn	Designer arrays for defined mutant analysis
DETA/NO	Diethylenetriamine/NO
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DOTS	Directly Observed Therapy Short-course
DTA	Dubos-Tween-Albumin
EHR	Enduring hypoxic response
FACS	Fluorescence Activated Cell Sorter
g	Gram
G	Guanine
GAF	Cyclic GMP, adenylyl cyclase, FhlA
gDNA	Genomic DNA
GFP	Green fluorescent protein

GMP	Guanosine monophosphate
h	Hour
HIV	Human immunodeficiency virus
HRP1	Hypoxic Response protein 1
IPTG	Isopropyl-ß-D-1-thiogalactopyranoside
IVET	*In vivo* expression technology
kb	Kilobase
kDa	Kilodalton
kV	Kilovolt
L	Litre
LB	Luria-Bertani
Lys	Lysine
M	Molar
MDR	Multi drug-resistant
mg	Milligram
MIC	Minimal Inhibitory Concentration
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MMT	Minimal medium
mRNA	messenger RNA
MTC	*Mycobacterium tuberculosis* complex
MU	Miller units
mut	Mutant
ng	Nanogram
nm	Nanometre
NRP1	Non-replicating phase stage 1
NRP2	Non-replicating phase stage 2
OADC	Oleic acid-albumin-dextrose-catalase
OD	Optical density
ONPG	Ortho-nitrophenyl-ß-galactoside
ORF	Open reading frame
PAS	Para-aminosalicylic acid

PCR	Polymerase chain reaction
qRT-PCR	Quantitative RT-PCR
rev	Reverse
RFU	Relative fluoresence units
RNA	Ribonucleic acid
rpm	Rotations per min
RT	Room temperature
RT-PCR	Reverse transcription PCR
s	Second
SDM	Site directed mutagenesis
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SPER/NO	SPERMINE NONOate
ssRA	Small stable RNA A
STM	Signature tagged mutagenesis
T	Thymine
TA	Toxin-antitoxin
TB	Tuberculosis
TraSH	Transposon site hybridisation
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
v/v	Volume per volume
vol	Volume
w/v	Weight per volume
WHO	World Health Organization
wt	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
XDR	Extremely drug-resistant
x g	Acceleration due to gravity
µF	Microfarads
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre

Ω	Ohm

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1. General Introduction

1.1 The genus mycobacterium

Mycobacteria are members of the Mycobacteriacea family, part of the Actinobacteria phylum. The mycobacterium genus comprises over 100 species dwelling in soil, water or found associated with plants, animals or humans (Primm *et al.*, 2004; Tortoli, 2006). The genus is divided into fast- and slow-growers. Slow-growers require at least one week to form colonies on solid media and include *Mycobacterium tuberculosis* and *Mycobacterium leprae*; the latter has not been successfully cultured *in vitro* (Goodfellow, 1982). In contrast, the fast-growers, such as *Mycobacterium smegmatis* only require two to three days to form colonies on solid medium. Mycobacteria are aerobic and have a rod structure (Draper, 1982). The general characteristics of mycobacteria include acid-fastness, a G/C-rich genome and a thick waxy coat mainly composed of high molecular weight mycolic acids (Clark-Curtiss *et al.*, 1985). The complex cell wall of mycobacteria is unique amongst prokaryotes, containing rigid peptidoglycan (PGN), arabinogalactan (AG), mycolic acid and over 60% complex lipids (Goude and Parish, 2008). Whilst most species are non-pathogenic environmental bacteria, some are highly successful pathogens, including *M. leprae* (Sasaki *et al.*, 2001), *Mycobacterium ulcerans* (Sizaire *et al.*, 2006), and the tuberculosis (TB)-causing species *M. tuberculosis* and *Mycobacterium bovis.*

1.1.1 *Mycobacterium tuberculosis* complex

The *Mycobacterium tuberculosis* complex (MTC) of TB-causing mycobacteria includes the species *M. tuberculosis, M. bovis, Mycobacterium africanum, Mycobacterium canetti* and *Mycobacterium microti* as well as the vaccine strain *M. bovis* Bacille Calmette-Guérin (BCG) (Zink *et al.*, 2003). Members of the MTC differ in morphology, host range, virulence and disease patterns (Brosch *et al.*, 2002; Frothingham and Meeker-O'Connell, 1998). *M. tuberculosis* is host-restricted, causing TB in humans and some primates, in contrast to the zoonotic *M. bovis* which causes disease in several types of mammal including humans and cattle (Smith *et al.*, 2006).

1.2 Tuberculosis

1.2.1 History and current global implications

Tuberculosis is an infectious disease that commonly affects the lungs (pulmonary TB), but it can also affect other parts of the body such as the central nervous system, bones, uro-genital tract and digestive system. In case of pulmonary TB, symptoms include coughing, night sweats, fever and rapid weight loss (Cole, 2005; Smith, 2003). Tuberculosis is believed to be a very ancient disease; archaeological evidence of bone TB has been identified in Egyptian mummies, skeletal remains from the Iron Age and the descriptions of the disease in historical documents date as far back as the seventh century BC (Smith, 2003; Zink *et al*., 2003; Taylor *et al.*, 2005). In 1882, Robert Koch identified *M. tuberculosis* as the causative agent of TB (Schluger, 2005) and to date *M. tuberculosis* is the major causative agent of TB in humans (WHO).

Current data from World Health Organization (WHO) shows that TB is a major health problem causing just under two million deaths each year (WHO, 2009). Annually, about nine million new cases of tuberculosis are reported globally and about one third of the world's population is estimated to be latently infected with this pathogen. In addition to this magnitude of disease reservoir, the WHO noted a 0.6% yearly increase in incidence worldwide, mostly due to new cases reported from the Asian and African regions, where the majority of cases are found (WHO, 2009). The increase in disease incidence has a large socio-economic impact and the estimated cost for TB control in countries with high disease prevalence is estimated to be $1.6 billion per annum (Frieden and Driver, 2003).

Until 1988, the disease occurrence in England and Wales was in decline. However, since then, incidence of the disease in the developed world has been increasing, which has been attributed to co-infection with HIV, immigration from high prevalence countries and social as well as economic deprivation (Antoine *et al.*, 2006). In particular, London appears to be a hot spot for TB, with the incidence rate of TB being five times higher than the rest of England and Wales. Indeed, 45% of the reported cases of TB in 2004 were in London. Within London, the boroughs of Hackney, Newham and Tower Hamlets have the highest incidence rates (60-80 cases per 100,000) (Antoine *et al.*, 2006).

1.2.2 Pathogenesis and immune response

The current clinical model of tuberculosis consists of three stages: primary infection (symptomatic, active disease), chronic infection (asymptomatic, latent disease) and post primary infection (reactivation of disease). Primary infection is initiated through inhalation of mycobacteria as aerosols from an actively infected patient. The inhaled mycobacteria are phagocytosed by alveolar macrophages in the lungs. Once inside the macrophage, the bacteria prevent fusion of the phagosome with lysosomes, thus escaping acidification, and replicate (Russell, 2001). They spread to the lymph nodes in the lung via the lymphatic system and form a lesion, known as the Ghon focus (Arany, 1959; Smith, 2003). Macrophages and lymphocytes are attracted to the site of infection and form a granuloma to isolate the infection and prevent further spread of the bacteria (Fig 1.1) (Cosma *et al.*, 2004; Dannenberg, 1994; Peters and Ernst, 2003; Saunders and Cooper, 2000), although recent studies suggest that the pathogen plays an active role in granuloma formation, and that the granuloma may actually function to aid mycobacterial dissemination during an infection (Davis and Ramakrishnan, 2009; Russell, 2007). Tuberculous granulomas are often caseous due to necrosis at the centre of the lesion and can release bacteria into the airways (Kaplan *et al.*, 2003). Tumor necrosis factor released by T-cells and infected macrophages is crucial for granuloma development, and the release of cytokines by differentiated macrophages directs the immune response to the site of infection (Dannenberg, 1994; Flynn and Chan, 2001). Cell-mediated immunity can contain the infection, and interferon gamma is thought to play a critical role in this process, but it is not always able to clear the infection (Schaible *et al.*, 1998). Surviving bacteria are believed to enter a period of non-replicating persistence somewhere in the body, and granulomas have been suggested as a possible location for this population of bacteria (Wayne and Sohaskey, 2001). Bacterial persistence during chronic or latent infection is one of the least understood aspects of tuberculosis, although latency is central to the prolonged presence of the pathogen in the host with the potential to give rise to reactivation of disease (Grange, 1992). People that are latently infected with TB are not contagious and do not show any symptoms of disease. Latent tuberculosis can reactivate after remaining dormant for many years and is a major health concern, because the antibiotics used to treat tuberculosis only work effectively on actively growing bacteria (Wayne

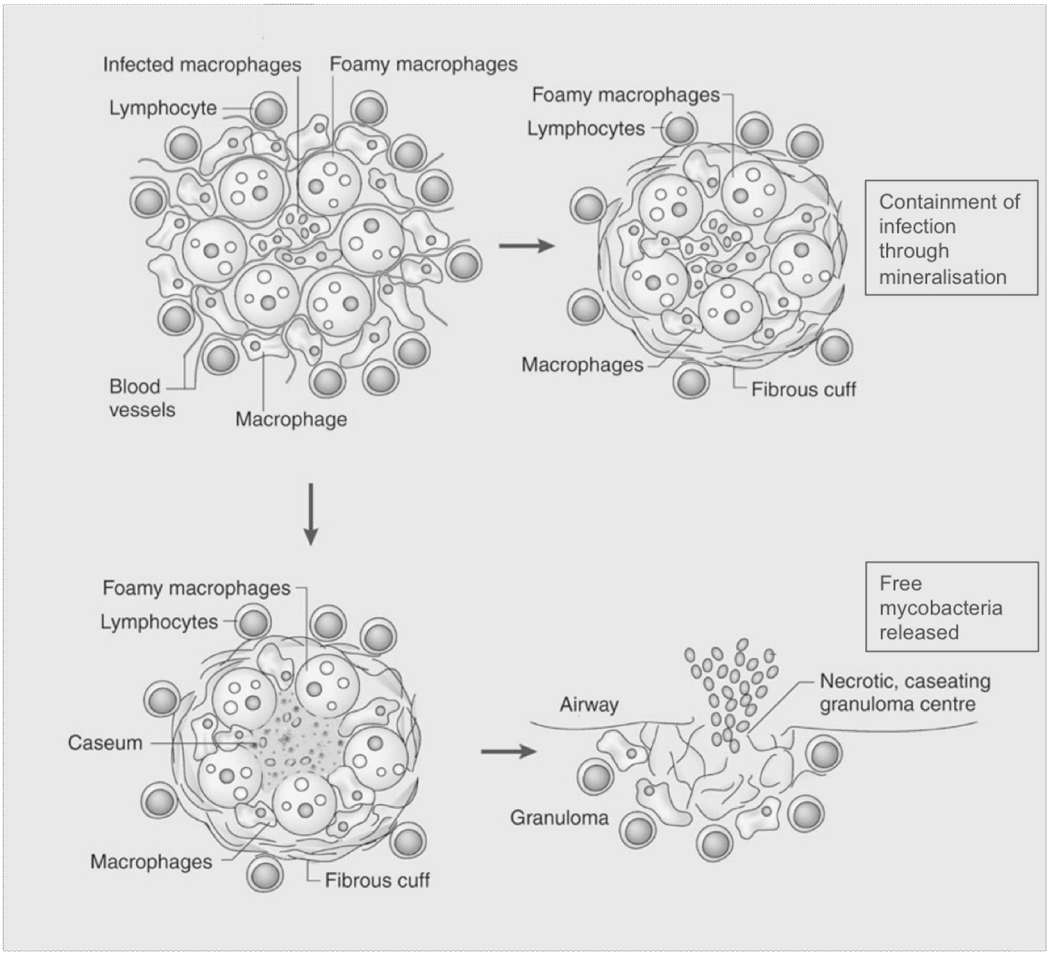

Figure 1.1. Schematic depiction of granuloma formation during an infection.

Figure adapted from Russell *et al.,* 2009.

and Hayes, 1996). Little is known about the signals that cause reactivation of the disease, but it has been shown that HIV-positive and immuno-compromised patients are more likely to develop overt disease (Garay, 1996; Tufariello *et al.*, 2006; Winthrop, 2006; Young *et al.,* 2005).

1.2.3 Models of mycobacterial persistence (*in vivo* and *in vitro*)

It has been difficult to determine how the bacteria survive for decades in their host without causing disease and the precise location of the persistent organisms within the human body has not been pinned down. There are several models that try to mimic the conditions that the bacterium encounters upon entering a persistent or dormant state during latent TB. Mycobacterial dormancy is defined as "a reversible state of low metabolic activity in which cells can persist for extended periods without division" and from which they can resume growth after resucitation (Kaprelyants *et al*., 1993). Models of persistence include *in vivo* mouse models such as the Cornell model, a model of chronic latency in the mouse and an artificial granuloma model (Karakousis *et al.*, 2004; McCune *et al.*, 1956; McCune and Tompsett, 1956a and b; Orme and Collins, 1994; Phyu *et al.*, 1998; Scanga *et al.*, 1999, Young *et al.,* 2005). Whilst these are useful for determining mycobacterial virulence factors of the mycobacteria, it has been difficult to recover sufficient bacteria to characterise the biochemical and physiological changes. The mouse model is widely used for the study of tuberculosis, but alternative animal models include the use of guinea pigs, rabbits and non-human primates (Dharmadhikari and Nardell, 2008). There are also *ex vivo* models in which cultured murine macrophages or human monocyte-derived cell lines are used (Biketov *et al.*, 2000; Paul *et al.*, 1996; Turner *et al.*, 2002; Young, 2005; Zhang *et al.*, 1998).

A variety of *in vitro* models have also been employed to unravel the nature of the persisting organisms by submitting them to conditions that they are believed to encounter during persistence, for example hypoxia - tuberculous lesions have little access to oxygen and their contents are essentially microaerobic or anaerobic (Aly *et al.*, 2006). Models of nutrient starvation, oxygen deprivation (for example the Wayne model of hypoxia), as well as prolonged stationary phase have allowed experimental assessment of the physiology and biochemistry of non-replicating bacteria

(Betts *et al.*, 2002; Hu *et al.*, 2000; Shleeva *et al.*, 2002; Sun and Zhang, 1999; Wayne and Hayes, 1996; Wayne and Sohaskey, 2001; Zhang *et al.*, 2001).

1.2.4 Vaccination strategies and treatment

Vaccination strategies

BCG is currently the only available vaccine against TB. This live attenuated strain of *M. bovis* was generated by French scientists Calmette and Guérin by passaging a virulent isolate for 13 years in a broth containing glycerol, potato-extract and bile salts. The isolate progressively lost its virulence and BCG was introduced as a vaccine against human TB in 1922. It is the most widely used vaccine in humans (WHO, 2004). Early clinical trials in Europe reported up to 80% protection, but subsequent studies showed variable efficacy (Brewer, 2000; Colditz *et al.*, 1994). Only 5% of all potentially vaccine-preventable deaths due to TB are thought to be prevented by BCG (Gupta *et al.*, 2007). Whilst this vaccine has clear beneficial effects against childhood TB, it only offers limited protection against the disease for a limited number of years. Several hypotheses have been put forward to explain this variability in the protective efficacy of BCG. Proposed reasons include differences amongst the vaccine strains used in clinical studies, exposure of trial populations to environmental mycobacteria or other infections that may affect mounting of a protective immune response to the vaccine, as well genetic and nutritional differences in human populations and trial methods (Liu *et al.*, 2009). Despite the fact that BCG is the most widely used vaccine in the world, TB is an ever increasing global problem. A major international research effort to develop new TB vaccines has been underway for many years, but a better alternative to BCG has yet to be produced (Delogu and Fadda, 2009).

Treatment of TB in humans

Administration of a single drug to treat a bacterial infection can lead to development of resistance. Therefore, effective treatment of TB involves multiple drugs to which the organisms are susceptible. Directly Observed Therapy Short-course (DOTS) antibiotic treatment can achieve high cure rates, such as an 85% global success rate reported by the WHO in 2007 (WHO, 2009). This efficient course of chemotherapy involves a combination

of four drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) which are taken for two months (intensive phase), followed by four months of isoniazid and rifampicin (continuation phase) (WHO, 1997). The success of this regimen relies on good compliance. As recommended by the WHO, DOTS should involve observing the patient taking every dose of the treatment, as well as maintaining detailed records of TB cases and following-up on patients, people whom they are in close contact with and individuals who discontinue the treatment. However, the cost of DOTS and the need for necessary infrastructure required to follow it, prevent its successful implementation in less economically developed countries. Patients often do not complete the full course of chemotherapy and poor compliance leads to high rates of treatment failure as well as the development of drug resistant strains (WHO, 2009).

Drug resistance

Despite drug efficacy, multi drug-resistant (MDR) and extremely drug-resistant (XDR) strains are becoming increasingly prevalent (WHO, 2009). MDR strains are resistant to isoniazid and rifampicin (both first line antitubercular drugs). XDR strains have resistance to isoniazid and rifampicin, plus resistance to one of the fluoroquinolones and either amikacin, kanamycin or capreomycin. Cure rates for patients with XDR are relatively low, especially if patients are co-infected with HIV. For example, in an outbreak of XDR-TB in KwaZulu-Natal, South Africa, 98% of infected patients died (Gandhi *et al.*, 2006). In 2007, 3.1% of all new cases of TB were caused by MDR strains, 85% of which were accounted for by 27 countries, 15 of which were in the European region. By the end of 2008, 55 countries had reported at least one case of XDR-TB. Poor TB control, lack or misuse of drugs, poor follow-up and outdated TB control strategies are believed to have contributed to the emergence of MDR and XDR strains (WHO, 2009).

The development of drug resistance mechanisms in *M. tuberculosis* has been extensively studied. The genes responsible for resistance to first line antibiotics (isoniazid, rifampin, pyrazinamide and ethambutol), as well as second line antibiotics (amikacin, kanamycin, ciprofloxacin, ofloxacin, clarithromycin, ethionamide, cycloserine and streptomycin), have been identified by a number of researchers (Jain and Mondal, 2008; Riccardi *et*

al., 2009; Zhang and Yew, 2009). In the past 40 years, no drug with a novel mechanism of action has been released onto the market. However, the increased clinical burden of TB and emergence of MDR and XDR strains has renewed interest in the development of new drugs and there is an urgent need for the identification of new drug targets.

1.3 Drug discovery

In general, the discovery and development of new drugs is a complex, expensive and lengthy process. There are several steps involved in drug discovery, starting with target identification, followed by target validation, assay development and high-throughput screening, confirming hits to leads through to lead optimisation and development of a candidate (Showalter and Denny, 2008). Development of new antitubercular drugs is hindered by the limited repertoire of validated drug targets. Appropriate drug targets are genes involved in processes that are critical for survival during growth or infection. Potential targets for new drugs need to be defined in the context of their essentiality for survival *in vitro* and *in vivo*. Whilst a gene might be essential for growth during an infection, it might be dispensable for mycobacterial survival in defined medium and *vice versa* (Sassetti and Rubin, 2003). Thus, in order to identify new drug targets, essential genes need to be selected, their function identified and individual genes validated as potential drug targets. In addition to essentiality, other properties such as selectivity, vulnerability and suitability for structural studies also play important factors in the decision whether a target is worth pursuing for drug development.

1.3.1 Identification of essential genes

Disruption of an essential gene through mutations or deletions generates a lethal phenotype. High-throughput generation of random mutations can be employed to rapidly identify essential genes as well as genes important for virulence. Technologies involving rapid, random mutagenesis of a gene pool that were originally developed for *Salmonella*, including *in vivo* expression technology (IVET) or signature tagged mutagenesis (STM) have been adapted for mycobacteria and allowed for study of phenotypes of mutant strains in which genes have been disrupted through random insertion of transposons (McAdam *et al.*, 1995; Tyagi, 2000).

There are several techniques that can be used to screen large pools of mutants for essential genes, for example the construction of a library of tagged mutants using transposon mutagenesis. Random insertion of a transposon results in disruption of a gene. If a gene is essential for bacterial survival, disruption of the gene would have a lethal phenotypic effect. Genes that are not disrupted in a large pool of mutants (i.e. non-essential genes) can be identified, and genes that cannot be mutated by transposon insertion can be predicted to be essential. Transposon mutants can be detected by several methods, for example by mapping the insertion sites of individual mutants by sequencing (Lamichhane *et al.*, 2003). Another technique, which is less limited by the current sequencing technology and that makes use of DNA microarrays, is transposon site hybridisation (TraSH) (Sassetti *et al.*, 2003). Using TraSH, a mutant pool is analysed by the generation of probes that hybridise to regions in the chromosome adjacent to the site of transposon insertion on a DNA microarray. This technique has been used to look at the essentiality of genes under defined growth conditions such as - on specific media, in macrophages or during a mouse infection model (Rengarajan *et al.*, 2005; Sassetti *et al.*, 2003; Sassetti and Rubin, 2003). Large pools of mutants can also be analysed by DeADMAn (designer arrays for defined mutant analysis), a technique in which *a priori* knowledge of transposon mutants grown *in vitro* is used to design specific probes that can be applied to characterise which mutants survive in a pooled *in vivo* infection (Lamichhane *et al.*, 2005).

1.3.2 Drug target validation

Once a potential drug target candidate has been identified by the screens described above, it needs to be validated, i.e. demonstrated that this molecular target is critically involved in the disease process and that modulation is likely to have a therapeutic effect. To do this, one needs to confirm its essentiality, identify its function and examine the effect of varying expression level in both *in vitro* and *in vivo* settings. Essentiality is a necessary but not sufficient requirement for a good drug target. One needs to evaluate under which conditions the gene is essential for growth, i.e. assay growth *in vitro*, for example by use of different media, carbon sources or exposure to hypoxic or iron-deplete conditions, as well as *in vivo*. A gene that is essential for *in vitro* growth might not be a good drug target if it is not essential for virulence or survival *in vivo*. Furthermore, even

if a gene has been shown to be instrumental for the disease process, target vulnerability - i.e. how much of the gene has to be inactivated before a therapeutic effect can be seen - is an important criteria for a good drug target. In addition to this, once a drug has been designed to target a certain gene product, one needs to be able to confirm that it specifically targets this product. Target specificity can be investigated by measuring resistance or sensitivity of bacteria to a drug in response to varying expression levels of the target gene or looking at the effect of a drug on gene expression by use of microarray or protein studies.

1.4 Current research tools

To find new drug targets one needs to identify genes that are important for mycobacterial growth, survival and pathogenesis. To single out such candidate genes one has to first gain a deeper understanding of the underlying genetics and biology involved. Second, one has to develop techniques to validate them as good drug targets by elucidating under what specific conditions the gene is essential to the organism.

Although the genome sequence of the H37Rv strain of *M. tuberculosis* was completed in 1998 (Cole *et al.*, 1998), only 52% of the predicted 3995 protein encoding genes have proposed functions (Camus *et al.*, 2002). Progress to identify the roles of the genes encoded by the genome has been hampered by a lack of genetic tools and the difficulty of working with *M. tuberculosis*, which is an extremely slowly growing organism that requires the use of specialised laboratories. Genetic systems that have been used widely in organisms such as *Escherichia coli* do not work well in mycobacteria. However, intensive research into electroporation and the use of replicative and integrative mycobacterial shuttle vectors has greatly aided the delivery of foreign DNA into the cells and advanced the development of genetic systems for mycobacteria (Ehrt and Schnappinger 2006; Jacobs *et al.*, 1991; Lewis, 2003; Machowski *et al.*, 2005; Parish *et al.*, 2001;).

Gobal genome analysis using microarrays has contributed to a more profound understanding of mycobacterial physiology. Transcriptional responses of *M. tuberculosis* to stress *in vitro* (such as exposure to heat shock, acid, hypoxia, nitric oxide, hydrogen peroxide, iron limitation or SDS), to a variety of metabolic inhibitors or *in* and *ex vivo* models have

been studied extensively (Boshoff *et al.,* 2004; Kendall *et al.,* 2004a). Recent advances in global genome analysis include the use of genome-directed primers to preferentially amplify mycobacterial genes and have allowed analysis of RNA extracted from small clusters of bacteria in infected tissues, giving an insight into global gene expression under those conditions. Expression of a particular gene of interest can be studied by techniques such as *in situ* hybridisation or quantitative real-time PCR or by use of reporter genes (Clark-Curtiss and Haydel 2003; Murry and Rubin 2005). The use of two-dimensional gel electrophoresis and the compilation of mycobacterial proteomic databases have helped to characterise and identify proteins unique to *M. tuberculosis*, as well as aided in the analysis of proteins produced in specific environments (Clark-Curtiss and Haydel, 2003; Murry and Rubin 2005).

Use of transposon mutant pools can predict gene essentiality, and use of the techniques outlined above can give insight into the function of a gene. However, essentiality of candidate genes and their potential as drug targets (i.e. properties such as target vulnerability and target specificity) need to be confirmed on an individual basis. This can be achieved by the use of several strategies such as the creation of knockout mutants, use of site specific recombination systems, antisense oligonucleotides and regulated promoters, which are described in the following section.

1.4.1 Construction of targeted deletion mutants

Disruption of an essential gene generates a lethal phenotype, and the inability to construct a targeted deletion mutant of a gene is generally accepted as evidence for gene essentiality. There are several different approaches for construction of a targeted mutant strain (Armitige *et al*., 2000; Balasubramanian *et al.,* 1996). Gene replacement through allelic exchange is a common approach and can be performed by either a two step homologous recombination strategy or a single step strategy (Bardarov *et al.,* 2002; Parish and Stoker 2000). Rates of homologous recombination can be increased by the use of phage proteins in a process called “recombineering” (van Kessel and Hatfull 2007; van Kessel and Hatfull 2008).

1.4.2 Antisense oligonucleotides

Successful inhibition of gene expression by the use of antisense oligonucleotides has been demonstrated in mycobacteria (Harth e*t al.,* 2000; Harth *et al.,* 2002; Harth *et al.,* 2007). This approach is based on gene silencing through the provision of an antisense strand of RNA which can bind to a sense strand of RNA (i.e. the mRNA) of a gene of interest and thus block its translation into protein. Harth and colleagues were able to silence genes by the addition of exogenous phosphorothioate-modified nucleic acids, thus demonstrating that the proteins they encode could be good drug targets. This approach was successful. However, the rationale behind the successful design of these antisense molecules and their exact mechanism of action is not fully understood. In addition to this, the system is limited for use in *in vitro* settings, due to the fact that administration of these molecules would be challenging during an infection model (Harth *et al.,* 2000; Harth *et al.,* 2002; Harth *et al.,* 2007; Wei and Rubin 2008). The use of regulated promoters for the provision of antisense RNA within mycobacterial cells is described in the following section.

1.4.3 Inducible promoter systems

Inducible promoters can be used to regulate expression of a gene - either in an on or off or a titratable setting - under controlled conditions. A promoter is a specific DNA sequence upstream of a gene to which the RNA polymerase binds and upon binding, initiates gene expression. Prokaryotic promoters consist of two short sequences at the -10 and -35 position upstream of the transcriptional start site. The *E. coli* consensus sequence consists of two hexamers: the Pribnow box (TATAAT) at -10 and TTGACA at -35 (Fig 1.2) (Alberts, 2002).

Inducible promoter systems have long been a genetic tool for studying gene function in microbiology, as they allow controlled regulation of expression of a gene of interest in response to a defined stimulus. Not only can they be used to overexpress a gene (for protein purification or phenotypic studies), but they can also be used to silence gene expression, thus providing an attractive way of studying essential genes. By definition, it is impossible to construct a mutant of a gene that is essential for growth. However, studying the phenotype of a conditional mutant may help to unravel under which growth conditions the gene is essential and thus give a

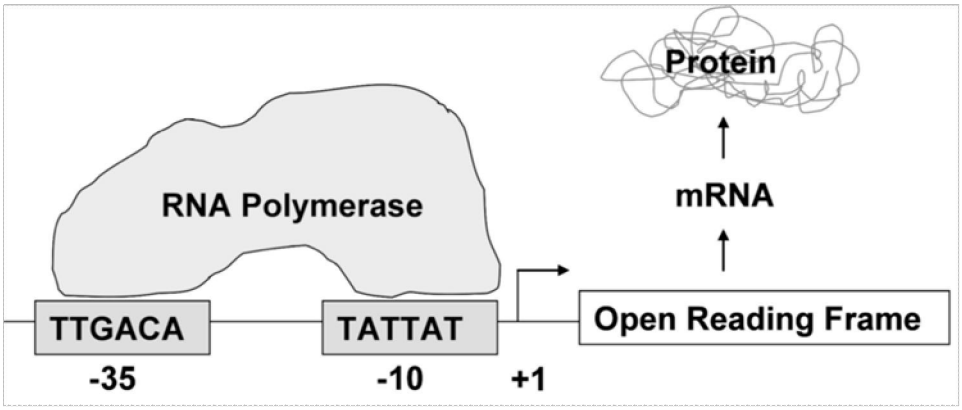

Figure 1.2. Schematic depiction of bacterial promoters.

This picture shows the *E. coli* consensus sequence of the -35 and -10 promoter elements to which RNA polymerase binds. The majority of mycobacterial promoters do not adhere to this consensus, and often there is no -35 element.

clue to its function. By use of an inducible promoter, one can regulate expression of the gene - either on/off or at varying levels, depending on the promoter system used - under defined conditions. If the inducible promoter system allows for titratable gene expression, one can also investigate target vulnerability, i.e. determine how much of the gene needs to be repressed before a detrimental effect on bacterial survival can be observed. Thus, dose-responsiveness is a property which makes inducible expression systems the ideal tool for validating the target of a drug, as resistance of the bacteria to a drug should correlate to the amount of target present in the cells.

Conditional knockdown mutants can be generated by a sense approach in which the native promoter of a target gene is replaced by one which is tightly regulated and inducible (Carroll *et al.,* 2005; Chalut *et al*., 2006; Ehrt *et al.,* 2005; Gandotra *et al.,* 2007; Guo *et al.,* 2007). Alternatively, an antisense approach can be employed in which controlled expression of antisense RNA from an inducible promoter leads to target gene silencing (Blokpoel *et al.,* 2005; Guo *et al.,* 2007). Gene expression systems that allow low or high levels of expression of mycobacterial genes during an infection are useful in the characterisation of genes involved in survival in the host as well as during mycobacterial persistence.

The ideal inducible expression system should be tightly switched off and should quickly respond to non-toxic concentrations of inducer or repressor, allow regulation over a range of expression levels and permit gene regulation during infection of cells *in vivo.* An array of inducible promoter systems, regulated by various stimuli such as tetracycline (P_{tet}), arabinose (P_{BAD}), IPTG and/or tryptophan (P_{lac}, P_{trp} and P_{tac}) has been developed for use in Gram-negative bacteria such as *E. coli* (Berens and Hillen 2003; de Boer *et al.,* 1983; Guzman *et al.,* 1995; Hillen and Berens 1994). Tetracycline-, fucose-, nisin- and IPTG-inducible promoters are amongst some that have also been developed or adapted for use in Gram-positive bacteria such as *Lactococcus*, *Staphylococcus*, *Streptomyces*, *Corynebacterium* and *Bacillus* species (Eichenbaum *et al.,* 1998; Stieger *et al.,* 1999; Zhang *et al.,* 2000; Bateman *et al.,* 2001; Chan *et al.,* 2003). In the past, several genetic systems have been developed to study function of *M. tuberculosis* and other mycobacterial genes using promoters that are induced in response to a specific stimulus.

The following section lists and describes the inducible promoter systems which are currently used for the study of mycobacterial genes.

The acetamide-inducible system

One of the earliest inducible promoter systems available for use in mycobacteria was the acetamidase system. It is based on the induction of the acetamidase gene of *M. smegmatis* by acetamide through a complex mechanism involving several promoters, two positive regulators of gene expression (AmiC and AmiD) and one repressor (AmiA) (Draper 1967; Mahenthiralingam *et al.,* 1993; Parish *et al.,* 1997; Parish and Stoker 1997; Parish *et al.,* 2001; Roberts *et al.,* 2003). The system has been used successfully for overexpression of mycobacterial proteins in *M. smegmatis* (Daugelat *et al.,* 2003; Triccas *et al.,* 1998). The systems was also used for construction of conditional mutants to confirm gene essentiality as well as to elucidate gene function of *whmD*, *dnaA* or *ftsZ* in *M. smegmatis* (Dziadek *et al.,* 2003; Greendyke *et al.,* 2002). However, special medium is required for optimal growth and activity and the system suffers from genetic instability in *M. tuberculosis* and a lack of tight regulation, which is why it is rarely used for study of gene essentiality in this slow-growing mycobacterium, although it has been used for conditional overexpression of genes of interest (Brown and Parish 2006; Hahn *et al*., 2005; Kang *et al*., 2005; Lee *et al.,* 2008a, Manabe *et al*., 1999; Parish and Stoker 1997; Park *et al.,* 2008).

Tetracycline-inducible systems

There are five tetracycline-regulated systems available for use in both fast- and slow-growing mycobacteria (Blokpoel *et al.,* 2005; Carroll *et al.,* 2005; Ehrt *et al.,* 2005; Guo *et al*., 2007; Hernandez-Abanto *et al*., 2006). They are based on regulation of expression from a promoter through a tetracycline-responsive repressor (TetR). The origin of the repressor and the promoters used to express TetR vary between the systems, but the general principle of tetracycline-inducible gene expression is the same. In the absence of inducer, TetR binds to the operator region of the promoter, blocks transcription, and promoter activity is switched off. In the presence of tetracycline, a conformational change in the regulator relieves binding and the promoter is switched on (Fig 1.3A).

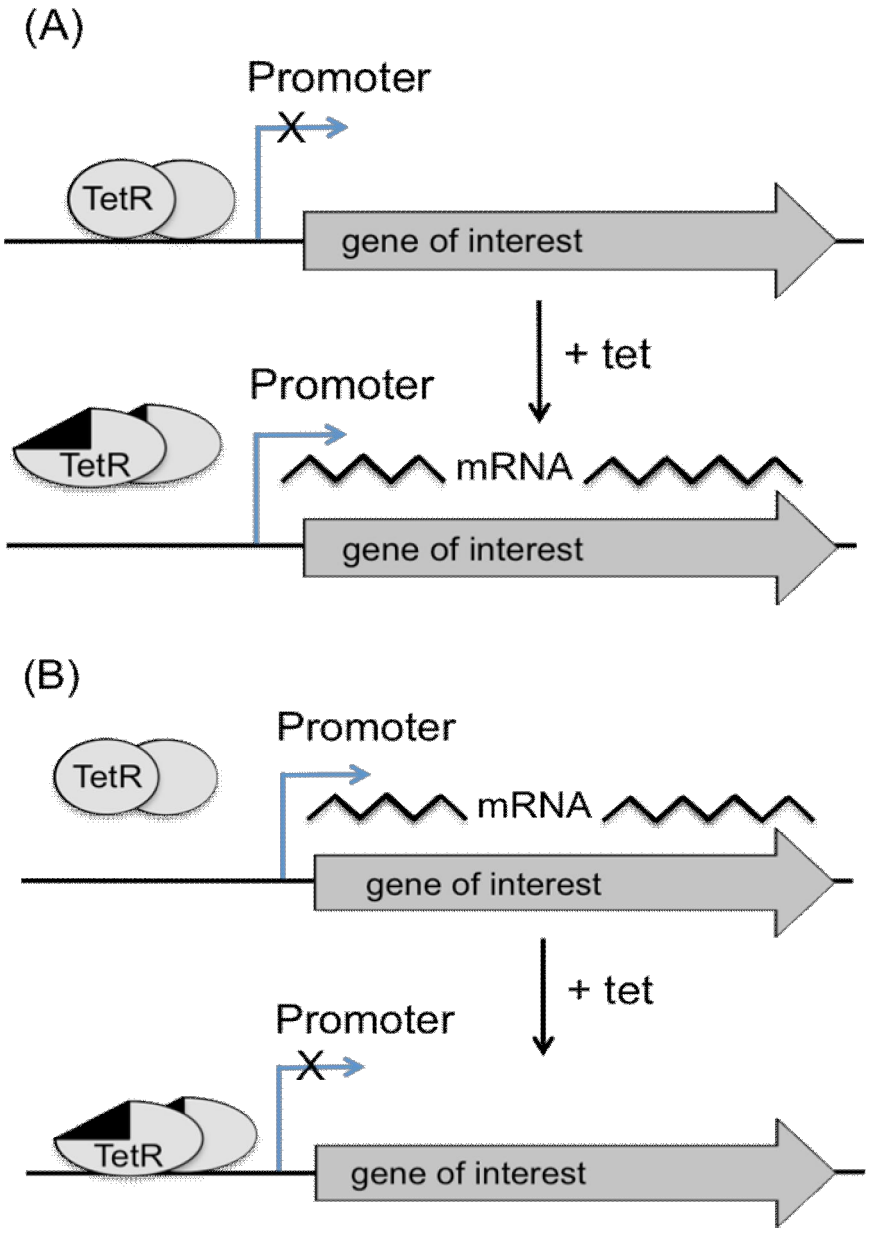

Figure 1.3. Regulation of gene expression through TetR in the Tet-ON and Tet-OFF systems.

(A) The Tet-ON system. In the absence of tetracycline, wild type TetR binds to the promoter region and blocks gene expression. In the presence of tetracycline, a conformational change of the repressor relieves binding and allows gene transcription. (B) The Tet-OFF system. In the absence of tetracycline, reverse TetR does not bind to the promoter region and allows gene expression. In the presence of tetracycline, a conformational change in TetR bound to tetracycline enables repression of gene expression.

Carroll *et al.,* 2005 developed a tetracycline-inducible system based on a Tn*10*-derived tetracycline regulatory system from *E. coli*, which had previously been adapted for use in *Bacillus subtilis* and *Staphylococcus aureus* (Geissendorfer and Hillen 1990; Ji *et al.,* 1999; Zhang *et al.,* 2000; Bateman *et al.,* 2001). The system uses TetR and $P_{xyl/tetO}$, a strong *B. subtilis* promoter with tet operator (tetO) integrated between the -35 and -10 hexamers. TetR was expressed from its own promoter, P_{tet}, or the constitutively active *M. bovis* BCG Antigen 85A promoter (P_{Atet}). They showed this system to be functional in mycobacteria using tagged *gfp* reporter genes linked to $P_{xyl/tetO}$. The system was dose-dependent and up to 5-fold and 10-fold inductions were achieved upon addition of tetracycline in *M. smegmatis* and *M. tuberculosis* respectively. Furthermore, induction was reversible by subculturing bacteria in tetracycline-free medium. A conditional auxotrophic mutant was constructed in *M. tuberculosis* by using the system to express *trpD* (a tryptophan biosynthetic enzyme) in an auxotrophic deletion mutant. However, even though this demonstrated that an inducible promoter could be successfully used for the construction of conditional mutants in *M. tuberculosis*, the system suffered from some basal expression in the absence of inducer.

Ehrt and colleagues also made use of the *E. coli* Tn*10*-encoded regulatory system, but instead of the $P_{xyl/tetO}$ promoter, a strong mycobacterial promoter ($P_{myc1tetO}$) was used (Ehrt *et al*., 2005). This promoter had been adapted for TetR binding by the insertion of two tet operators upstream of the -35 and -10 elements. Expression of TetR is controlled by one of two constitutively active mycobacterial promoters of varying strength (P_{smyc} and P_{imyc}). Instead of tetracycline, its less toxic derivative anhydrotetracycline is used as inducer. Dose-dependent induction of $P_{myc1tetO}$ activity in *M. smegmatis* was demonstrated by use of reporter genes - two different types of *gfp* and *lacZ*. The induction kinetics of the system were time-dependent and maximal induction was reached after four hours. The system was used to successfully construct an anhydrotetracycline-dependent conditional mutant of *M. smegmatis*: the native promoter of *ftsZ* (a bacterial tubulin homolog essential for cell division) was replaced with $P_{myc1tetO}$ in a strain that expressed TetR from P_{smyc}. Inactivation of FtsZ was confirmed by monitoring phenotypic effects in the absence of or presence of low concentrations of inducer. Functionality of the system was also

demonstrated in *M. tuberculosis* using *lacZ*. Stable induction of promoter activity (up to 160-fold) was observed 72 hours after addition of inducer. In addition to this, the system could also be used for anhydrotetracycline-regulated expression of *gfp* in *M. tuberculosis* during a macrophage infection. Even though this genetic system is functional in mycobacteria, similarly to the other Tn*10*-based system, some basal level of activity was detected in the absence of inducer (Ehrt *et al*., 2005). This system (Tet-ON system) was used for the construction of a conditional mutant in *M. bovis* BCG, and confirmed essentiality of *ppt* for *in vitro* growth (Chalut *et al.,* 2006).

In addition to the $P_{myc1tetO}$-TetR based Tet-ON system, there is also a Tet-OFF system which makes use of a reverse TetR (revTetR) mutant (Scholz *et al.,* 2004). RevTetR requires (anhydro)tetracycline as a corepressor (Fig 1.3B) and has been used for efficient gene silencing in *M. smegmatis* (Guo *et al.,* 2007). The system is dose-dependent and was used for construction of a conditional mutant to confirm essentiality of the *M. smegmatis* gene *secA1* (Guo *et al.,* 2007). The reverse TetR (Tet-OFF) system is useful for silencing gene expression under conditions where removal of an inducer through washes or subculturing proves to be challenging (for example hypoxic conditions, or during tissue culture or *in vivo* infections).

Both the Tet-ON and the Tet-OFF system have been used to construct conditional knockdown mutants in *M. tuberculosis* using a sense approach (Gandotra *et al*., 2007). The systems were used to silence and confirm essentiality of the mycobacterial gene *prcBA in vitro* and *in vivo*, demonstrating the functionality of the Tet-ON and the Tet-OFF system during a mouse infection (Gandotra *et al*., 2007). TetR efficiency in both systems was improved through codon optimisation of the wild type and reverse TetRs for mycobacteria (Klotzsche *et al*., 2009). Codon optimisation improved repression through wtTetRs 3-fold in *M. smegmatis* and up to 50-fold in *M. bovis* BCG. Repression through revTetRs was improved to 70-fold through a combination of codon optimisation and the introduction of amino acid mutation to improve efficiency of tetO binding. The improved revTetRs allowed efficient gene silencing of *secA1* in *M. smegmatis* although repression through the new revTetRs was not as efficient in *M. bovis* BCG (Klotzsche *et al*., 2009).

A third tetracycline-inducible system is based on the TetZ determinant of plasmid pAG1 of *Corynebacterium glutamicum* (Blokpoel *et al.,* 2004). This locus had previously been shown to control expression of a tetracycline efflux system in *C. glutamicum* (Tauch *et al.,* 2000). The system is based on tetracycline-dependent TetR(Z) repressor binding to the *tetRO* region, but neither the promoter nor the TetR binding sites of this region have been characterised (Blokpoel *et al.,* 2004). This system enabled dose-responsive and time-dependent induction of the *luxAB* reporter gene in both *M. smegmatis* (up to 70-fold induction) as well as the slow-growing mycobacteria *M. tuberculosis* (up to 10-fold induction) and *M. bovis* BCG (up to 20-fold induction). However, in the absence of inducer, the system was not tightly repressed. Despite this drawback, the system was successfully used to silence expression of *ftsZ* in *M. smegmatis* by tetracycline-regulated expression of an antisense RNA. In addition to this, use of the system also allowed induction of reporter gene expression in *M. bovis* BCG during a macrophage infection (Blokpoel *et al.,* 2004).

In addition to the aforementioned systems, there is a fourth tetracycline-inducible expression system which has been adapted from *Streptomyces coelicolor* for use in *M. smegmatis* (Hernandez-Abanto *et al.,* 2006). This systems is based on TetR-controlled regulation of a modified version of the promoter of the *S. coelicolor* gene *tcp830* whose -10 promoter element resembles that of strong mycobacterial promoters. The mutations enhanced induction of promoter activity and tight control of this system was achieved by regulating TetR expression through an acetamide-inducible promoter. The system was shown to achieve up to a 130-fold induction of GFP in *M. smegmatis* and was used in a murine intraperitoneal cavity infection model using *M. smegmatis* (Hernandez-Abanto *et al.,* 2006).

The pristinamycin-inducible system

An alternative to the tetracycline-inducible systems, which suffer from background activity, is the prinstinamycin-inducible system which has been adapted for use in mycobacteria (Forti *et al.*, 2009). The system is based on the pristinamycin I-responsive regulator Pip from *S. coelicolor* which binds to operator sites in the promoter region of the *Streptomyces pristinaespiralis* gene *ptr* (P_{ptr}) and thus represses gene expression in the absence of pristinamycin (Blanc *et al.,* 1995; Folcher *et al.,* 2001; Frey *et*

al., 2001, Fussenegger *et al.,* 2000). This inducible system has previously been shown to have a low basal level combined with a high induction rate and to be functional in both plant and mammalian cells (Fussenegger *et al.,* 2000; Frey *et al.,* 2001). Functionality of the system was tested in both *M. smegmatis* and *M. tuberculosis* by using P_{ptr} linked to the reporter gene *lacZ* and constitutive expression of Pip from mutated *M. tuberculosis furA* promoters $P_{furA102}$ or $P_{furA104}$, which differ in strength. P_{ptr} is a strong promoter and can be fully repressed by Pip in both mycobacteria. Upon addition of pristinamycin, Pip-mediated repression was relieved in a dose-responsive manner. Inductions of up to 50-fold and 160-fold were achieved by addition of low concentrations of inducer in *M. smegmatis* and *M. tuberculosis* respectively. The system was used to successfully overexpress proteins encoded by three mycobacterial genes (*glnA1*, *pknB* and *fadD32*) in response to pristinamycin in *M. tuberculosis*. Pristinamycin controlled overexpression of antisense RNA of these genes resulted in a partial knockdown of the genes at transcript and protein level (as measured by real time RT-PCR and Western analysis). A slight growth defect of knockdown mutants of *fadD32* and *pknB* was observed on solid medium. The system was also used to construct conditional mutants of *fadD32* and *pknB* by direct promoter replacement. These mutants failed to grow in the absence of inducer but grew well in the presence of inducer.

IPTG-inducible systems

Two IPTG-inducible systems of varying origin have also been adapted for use in mycobacteria (Lee *et al.,* 2008b; Kaur *et al.,* 2009).

Lee and colleagues constructed an IPTG-inducible green fluorescent reporter strain of *M. tuberculosis* for studying metabolic activity of bacteria during growth in macrophages (Lee *et al.,* 2008b). The system is based on the T7 lac repressor/operator system from *E. coli* and consists of the IPTG-responsive repressor $LacI^q$, expressed from a strong constitutively active *M. tuberculosis* promoter (P_{glnA1}), and its cognate promoter, P_{T7lac} which was linked to the reporter gene *gfp*. IPTG-controlled expression from P_{T7lac} was mediated through T7 RNA polymerase which was also expressed from the constitutively active promoter of the 32 kDa mycolyl transferase gene of *M. tuberculosis* (P_{32}). In the presence of IPTG, LacI repression of P_{T7lac} was relieved and *gfp* expression could be initiated through T7 RNA polymerase.

The system was shown to achieve high induction of GFP (up to 40-fold after 1-3 days) in both extracellular and intracellular bacteria, even though low background activity was observed in the absence of inducer. This system was used to demonstrate that metabolically active bacteria (expressing *gfp* in response to IPTG treatment) resided in non-acidified phagosomes, whereas metabolically inactive bacteria were found in acidified phagosomes only.

Kaur and colleagues adapted an IPTG-inducible Lac system from *E. coli* for use in mycobacteria and used it for antisense inhibition of mycobacterial genes in order to delineate whether the effect of their silencing on growth kinetics was bacteriostatic or bacteriocidal (Kaur *et al.*, 2009). To ensure that the system would work equally well in fast- and slow-growing mycobacteria, a *lac* operator was cloned downstream of P_{trc} which contains a promoter close to the mycobacterial SigA consensus sequence (Unniraman *et al.*, 2002). LacI repressor was constitutively expressed from the moderately active T150 promoter which has previously been shown to be equally potent in *M. smegmatis*, *M. tuberculosis* and *M. bovis* BCG (Bashyam *et al.*, 1996). Functionality of the system was tested by means of the reporter gene *lacZ* in *M. smegmatis*. An induction of up to 30-fold was seen after four hours and the level of leaky expression in the absence of inducer was low, even though accumulation of reporter gene as a result of this lack of tight regulation was observed after 12 hours. Proof of principle that this system could be used to inhibit expression of a target gene through controlled expression of antisense RNA was achieved by successful use of the system for construction of a conditional antisense knockdown of FtsZ in *M. smegmatis*. Using this system, conditional antisense knockdowns of *gyrA*, *gyrB*, *inhA*, *embB*, *rpoB*, *rpoC*, *rplJ*, *rpsL* as well as *ilvB* were made and their survival kinetics were monitored in *M. smegmatis* and *M. tuberculosis*.

Caprolactam- or nitrile-inducible systems

Kang and colleagues demonstrated the utility of a caprolactam-inducible promoter system for regulated expression of genes of interest in *M. smegmatis* (Kang *et al.,* 2005). The system is based on an inducible nitrilase expression mechanism from *Rhodococcus rhodochrous* and has been developed for use as a hyper-inducible expression system in

Streptomyces (Herai *et al.,* 2004). The system consists of the positive regulator NitR which activates expression of the P_{nitA} promoter in the presence of caprolactam. NitR as well as the target gene are expressed from P_{nitA}. The system is dose-dependent, but some expression of NitR occurs in the absence of inducer (Herai *et al.,* 2004). Kang and colleagues used this system for inducible antisense knockdown of the essential genes *pknA* and *pknB* in *M. smegmatis* (Kang *et al*., 2005).

Even though NitR can be activated by a range of inducers in *R. rhodochrous*, only a subset of these work in mycobacteria, and caprolactam is not a potent inducer in slow-growing mycobacteria (Pandey *et al*., 2009). Pandey and colleagues demonstrated that the nitrile isovaleronitrile can be used to activate NitR-mediated expression of a reporter gene (*gfp*) from P_{nitA} over 100-fold in both *M. smegmatis* and *M. tuberculosis*. In addition to this, the system was also functional in intracellular *M. smegmatis* and shown to have similar fold induction and induction kinetics to the Tet-ON system (Ehrt *et al*., 2005). However, unlike the TetR system, this system acts as a bistable switch (i.e. 50% of the cell population will express a target gene and the other 50% will not).

Limitations of inducible expression systems

The perfect inducible promoter system should have the following properties: it should be functional in both fast- and, more importantly, slow-growing mycobacteria; it should respond to low concentrations of stimulus, be tightly regulated and, if used for drug target identification or validation, allow titratable gene expression and be functional *in vivo*. Even though some of these criteria are fulfilled by the currently available systems - they all respond to non-toxic concentrations of inducer - none of these system possess all the desired properties. For example, the acetamide-inducible system is unsuitable for use *in vivo* (Ehrt and Schnappinger, 2006). The (anhydro)tetracycline-inducible systems are suitable for *in vivo* work, but they suffer from a lack of tight regulation; although in the case of the Tet-ON and Tet-OFF system, this has been improved by the development of codon optimised TetR regulators (Ehrt *et al*., 2005; Chalut *et al*., 2006; Gandotra *et al*., 2007; Guo *et al*., 2007; Klotzsche *et al.,* 2009). The pristinamycin-inducible system is tightly regulated, with little to no expression in the absence of inducer, but has yet to be shown to be

functional *in vivo* (Forti *et al.,* 2009). Both IPTG-inducible systems work well in fast- and slow-growing mycobacteria and the system developed by Lee and colleagues is functional during macrophage infection (Lee *et al*., 2008b). However, both IPTG-regulated systems suffer from background activity in the absence of inducer (Lee *et al*., 2008b; Kaur *et al.,* 2009). The titratable caprolactam-inducible system developed by Kang and colleagues functions in fast-growing mycobacteria, but the inducer is not suitable for use in slow-growers (Kang *et al*., 2005, Pandey *et al*., 2009). The system developed by Pandey and colleagues, which uses nitrile as an an alternative inducer, does not regulate gene expression in a titratable manner (Pandey *et al*., 2009).

The expression systems currently available have proved useful in the construction of conditional mutants to confirm gene essentiality or elucidate gene function. However, lack of tight regulation is a crucial limitation; as this would be problematic for the study of genes which are naturally expressed at a very low level. The use of a leaky promoter would cause the gene to be artificially overexpressed in the supposedly “uninduced” state, and essential genes might be masked by this effect. In addition to this, if one wished to study the effect of conditional silencing or overexpression of more than one gene within a strain, it would be advantageous to make use of more than one expression system, in order to study the effects of expressing varying levels of more than one gene. Hence, there is a need for alternative expression systems which fulfill the criteria of tight regulation in slow-growing mycobacteria and *in vivo* functionality.

One feature that all of the existing systems have in common is that they are based on the adapatation of existing expression systems for use in mycobacteria by use of mycobacterial(-like) or modified promoters. And all, except for the pristinamycin-inducible system, suffer from a lack of tight regulation to varying degree. This particular problem could be circumvented by the use of an inducible expression system originating from mycobacteria, based on an inducible endogenous promoter, as this eliminates the requirements of modification of the promoter(s) or regulator(s) for use in mycobacteria. Whilst one might argue that use of an endogenous expression system will inadvertendly affect expression of genes other than one wishes to study, such a system might be regulated more tightly and work more efficiently in mycobacteria than the systems

currently available. Moreover, since such an endogenous system has not been developed yet, it would be interesting to test if it such a system could indeed be found and provide an alternative to the existing expression systems.

1.5 Mycobacterial promoters

In contrast to most prokaryotic promoters, mycobacterial promoters are G/C rich and very diverse (Gomez, 2000). There appears to be a poor conservation of the -35 element and they are classified into four groups. Group A has a both a -10 and -35 motif similar to the *E. coli* consensus, Group B is characterised by the presence of a -10 hexamer similar to the consensus, Group C has no resemblance to the consensus in either elements and Group D has a conserved -10 element and a -35 element clearly different from the consensus. In some mycobacterial promoters, deletion of the region upstream of the -10 hexamer leads to loss of transcriptional activity (Gomez, 2000). Another determinant of promoter activity in bacterial promoters, the extended -10 promoter, is also present in mycobacteria (Bashyam and Tyagi, 1998). The sequence TGN located directly upstream of the -10 element can enhance transcriptional strength of the -10 element (Bashyam and Tyagi, 1998).

Mycobacterial promoters or transcriptional active DNA sequences can be identified and characterised by means of reporter genes (Tyagi, 2000). There is a large repertoire of reporter genes (*cat, lacZ, phoA, xylE, lux* and *gfp*), but *lacZ* and *gfp* are two of the most commonly used, and several promoter probe vectors developed for mycobacteria make use of these two reporter genes (Blokpoel *et al.,* 2003; Carroll *et al.,* 2005; Ehrt *et al.,* 2005; Machowski *et al.,* 2005).

The *lacZ* gene of *E. coli* encodes the enzyme ß-galactosidase which catalyses the cleavage of lactose to liberate the monomeric sugars galactose and glucose (Fowler and Zabin, 1983). Cleavage of the lactose derivative ortho-nitrophenyl- ß-galactoside (ONPG) by ß-galactosidase gives rise to a yellow product (ortho-nitrophenol) which can be quantified by measuring the absorbance at 420 nm (Miller, 1972).

Green fluorescent protein (GFP) encoded by *gfp* of the jellyfish *Aequorea victoria* is a popular reporter gene. *Gfp* has been used to identify

mycobacterial promoters that are active during macrophage infection (Barker *et al.,* 1998). Unstable variants have been developed by addition of a peptide tail that targets the protein for degradation, and fusion vectors of unstable GFP are commonly used to study promoter activity (Blokpoel *et al.,* 2003; Carroll *et al.,* 2005).

1.6 Aims and objectives

Identification of putative drug targets will advance development of antimycobacterial agents. These could be used to tackle the problem of resistance against antibiotics which are currently in use and if they target a gene important for mycobacterial survival during latent disease, they may help to shorten the time course of treatment. The purpose of this work is to contribute to the discovery of new drug targets by development and use of novel inducible promoter systems. An expression system based on the use of inducible promoters endogenous to *M. tuberculosis* might be a good alternative to the existing systems, which have limited functionality in slow-growing mycobacteria such as *M. tuberculosis*. The aim of this study was to identify and characterise candidate promoters in *M. tuberculosis*, verify inducible expression and use these to express mycobacterial genes in a controllable fashion. The objectives of this project were:

- to look at a variety of conditions that have been shown to induce specific genes (e.g. induction by a specific stress) and identify their promoters;
- to assay these promoters using reporter genes to determine whether differential expression really occurs *in vitro* and *in vivo* and thus identify a panel of suitable promoters from which genes can be expressed in any given setting; and
- to test the most promising promoter using mycobacterial genes by generating knockdown strains using both antisense and sense approaches.

2. Materials and methods

Unless otherwise stated all chemical reagents were obtained from Sigma®-Aldrich (Sigma), Poole, United Kingdom (UK). Buffers and solutions were sterilised by autoclaving and antibiotics and chemicals added to bacteria were dissolved in sterile distilled water (SDW) unless otherwise stated and filter-sterilised.

2.1 Bacterial growth

2.1.1 Media

E. coli was grown at 37°C with shaking at 225 rpm in Luria-Bertani (LB) broth or on Luria-Bertani agar. Ampicillin sodium salt was used at 100 mg L^{-1}, gentamicin sulphate salt at 20 mg L^{-1}, hygromycin B (Roche Diagnostics) at 100 mg L^{-1}, kanamycin sulphate at 50 mg L^{-1}, streptomycin sulphate salt at 50 mg L^{-1}, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) (Melford Laboratories Ltd.) was dissolved in dimethyl sulfoxide (DMSO) and used at 50 mg L^{-1}.

M. smegmatis mc^2155 was grown at 37°C with shaking at 100 rpm (unless otherwise stated) in Lemco medium, which was prepared as follows: 10 g L^{-1} Bacto™ peptone (Beckton Dickinson), 5 g L^{-1} 'Lab-Lemco' powder (Oxoid), 5 g L^{-1} NaCl; containing 0.05% w/v Tween®80 for liquid cultures and 15 g L^{-1} Bacto™ agar (Beckton Dickinson) for solid media. Gentamicin was used at 10 mg L^{-1}, hygromycin B at 100 mg L^{-1}, kanamycin at 20 mg L^{-1}, streptomycin at 20 mg L^{-1} and X-gal at 50 mg L^{-1} when required.

M. tuberculosis H37Rv was grown at 37°C in Difco™ Middlebrook 7H9 medium (Beckton Dickinson) supplemented with 10% v/v BBL Middlebrook™ oleic acid-albumin-dextrose-catalase (OADC) supplement (Beckton Dickinson) and 0.05% w/v Tween®80 or on Difco™ Middlebrook 7H10 agar (Beckton Dickinson) supplemented with 10% v/v OADC unless otherwise stated. Cultures were grown without agitation in 50 mL tubes (Starstedt) unless otherwise stated. Gentamicin was used at 10 mg L^{-1}, hygromycin B at 100 mg L^{-1}, kanamycin at 20 mg L^{-1}, streptomycin at 20 mg L^{-1} and X-gal at 50 mg L^{-1} when required.

For mycobacterial growth under hypoxic conditions, Dubos-Tween-Albumin (DTA) medium was prepared according to manufacturer's instruction. In

180 mL purified water, 1.3 g of Dubos broth base (Difco, Beckton Dickinson) was dissolved and autoclaved. 20 mL of Dubos Medium Albumin (Difco, Beckton Dickinson) and 0.05% w/v Tween®80 were added aseptically.

For mycobacterial growth under conditions of low iron, minimal medium (MMT) was prepared as follows: 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl and 0.0147 g L^{-1} $CaCl_2$ supplemented with 0.05% w/v Tween® 80 and 2% v/v glycerol and treated overnight with 5 g L^{-1} Chelex 100. Before use, 2 mM $MgSO_4$ was added and the medium filter-sterilised.

2.1.2 Growth of *M. smegmatis*

Cultures of *M. smegmatis* were prepared as follows: a loopful of cells from a plate was used to inoculate 5 mL of medium, and grown overnight; cultures were diluted 1/10 to set up a secondary 5 mL culture. Unless otherwise stated, these were grown overnight or to mid-exponential phase before being subjected to inducing conditions as described for *M. tuberculosis* below.

2.1.3 Growth of *M. tuberculosis*

All work involving *M. tuberculosis* was carried out in a Containment Level 3 laboratory equipped for Advisory Committee on Dangerous Pathogens (ACDP) Hazard Group 3 work.

Preparation of seed cultures

To prepare seed cultures of *M. tuberculosis,* a loopful of cells from a plate was used to inoculate 10 mL medium containing 3 mL of 1 mm glass beads (BioSpec Products Inc.) and incubated without agitation for 14 d. 1 mL of this culture was used to inoculate 10 mL medium and grown without agitation for 7 d or until mid-log phase was reached as indicated by OD_{580} of 0.5-0.8.

Growth of mycobacteria for assaying promoter activity in response to antibiotics

To assay promoter activity in response to antibiotics, a seed culture was used to set up 10 mL medium with an OD_{580} of 0.1. Liquid cultures were grown to mid-log phase. Liquid cultures were subjected to treatment with 10 µg mL^{-1} of tetracycline hydrochloride (dissolved in DMSO) for 6 h, 50 ng

mL^{-1} tetracycline for 24 h, 7.5 µg mL^{-1} streptomycin for 3.5 h or 50 ng mL^{-1} streptomycin for 24 h and cell-free extracts were prepared.

Growth of mycobacteria for assaying promoter activity in response to ethanol or sodium dodecyl sulphate

To assay promoter activity in response to ethanol and sodium dodecyl sulphate (SDS), a seed culture was used to set up 10 mL liquid cultures with an OD_{580} of 0.1. These were grown to mid-log phase. Liquid cultures were subjected to treatment with 2.5% v/v ethanol for 3.5 h or 0.05% v/v SDS for 3.5 h and cell-free extracts were prepared.

Growth of mycobacteria for assaying promoter activity in response to stress conditions

To assay promoter activity under stress conditions, 3 mL of a seed culture were used to inoculate 100 mL medium in a 490 cm^2 roller bottle (Corning). The OD_{580} was measured after 72 h and diluted to an OD_{580} of 0.1. From this stock, 8 mL aliquots were made into 20 mm x 125 mm round bottom screw cap glass tubes and stirred with 3 x 8 magnetic stirring bars at 150 rpm (unless otherwise stated) for 7 d or 72-96 h until the OD_{580} was ~0.5.

Bacteria growing in liquid culture were subjected to the following stress conditions: standing culture (not stirred), 5% v/v ethanol and 50 µM diethylenetriamine/NO (DETA/NO). Cell-free extracts were prepared after 30 min in *M. smegmatis* and after 60 min in *M. tuberculosis*.

Growth of mycobacteria during the Wayne model of hypoxia

Liquid cultures were grown in Dubos-Tween-Albumin (DTA) in round bottom screw cap glass tubes of 20 mm x 125 mm (Fisher). To create a theoretical starting OD_{580} of 0.004, 170 µL of a culture with an OD_{580} of 0.3-0.4 was used to inoculate 17 mL medium. Caps were tightly screwed on and sealed with tape. Cultures were grown stirring at 150 rpm with a 3 x 8 mm magnetic stirring bar. Methylene blue (1.5 µg mL^{-1}) was used as a visual indication of oxygen depletion in a control tube. Fading of the methylene blue dye indicated onset of microaerophilic conditions (Non-replicating Phase stage 1) and decolourisation indicated onset of anaerobic conditions (Non-replicating Phase stage 2).

Growth of mycobacteria for growth profiles of promoter activity

For growth profiles, 100 mL of medium in a roller bottle was inoculated with 3 mL of a seed culture and incubated rolling at 37°C for 72 h. The OD_{580} of the roller culture was measured and 50 mL cultures with an OD_{580} of 0.1 were prepared. From this stock, 8 mL of culture was aliquoted into 20 x 125 mm round bottom screw cap glass tubes and stirred at 150 rpm with 3 x 8 mm magnetic stirring bars. The OD_{580} of cultures was measured and cell-free extracts were prepared every 4-7 d.

Growth of mycobacteria for assaying promoter activity in response to salicylate and related compounds

To assay promoter activity in response to salicylate in *M. tuberculosis*, 100 mL roller cultures were inoculated with 3 mL of a seed culture and grown until mid-log phase (as measured by an OD_{580} of 0.6 and 0.8). 10 mL aliquots were made and subjected to treatment. To assay promoter activity in *M. smegmatis* (or when only two growth conditions were being compared in *M. tuberculosis*), a seed culture was used to set up 10 mL liquid cultures with a theoretical OD_{580} of 0.1 and grown to mid-log phase. Liquid cultures were subjected to treatment with salicylate or structurally related compounds.

Mycobacteria grown in liquid cultures were treated with 0-10 mM of sodium salicylate. Cell-free extracts were prepared after 2 h or up to 21 d after addition of salicylate. The following compounds were used at 0.4 mM to treat *M. tuberculosis* liquid cultures for 3 d: acetylsalicylic acid (aspirin) (dissolved in DMSO), benzoate (dissolved in DMSO), fenofibrate (dissolved in DMSO), gemfibrozil (dissolved in ethanol), menadione (dissolved in DMSO) and para-aminosalicylic acid (PAS) (dissolved in DMSO).

Growth of mycobacteria for assaying promoter activity under iron depletion

To assay promoter activity in response to low iron, liquid cultures were grown in MMT. To achieve an iron-depleted state, bacteria were passaged in MMT several times (Rodriguez *et al.*, 2002). To set up a passage of bacteria in MMT, a mid-log phase culture was washed in 10 mL MMT and resuspended in 10 mL MMT. 1 mL of this was used to inoculate either a 10 mL liquid culture or a 100 mL roller culture. Passages were grown to an

OD_{580} of ~1.0 before 1 mL was used to inoculate the next passage and cell-free extracts were prepared.

Assaying growth curves of mycobacteria

Growth curves in *M. tuberculosis* were carried out in 4 mL medium in 16 x 125 mm round bottom screw cap glass tubes with a starting OD_{580} of 0.02. Cultures were stirred with 3 x 8 mm magnetic stirring bars at 150 rpm.

2.2 Extraction, purification and quantification of genomic DNA, RNA and complementary DNA

2.2.1 Extraction and purification of genomic DNA

M. tuberculosis cells from a 10 mL liquid culture were harvested by centrifugation for 10 min at 2700 x g, resuspended in 1 mL 10 mM Tris-Cl pH 7.5, and added to 2 mL lysing matrix B tubes (MP Biomedicals) on ice. Cells were disrupted using one 20 s cycle at speed 4.0 using a FP120 FastPrep™ (Fisher). Samples were spun at 16000 x g for 4 min, the supernatants were recovered and filter-sterilised through a 0.2 µm filter unit. This was followed by addition of 0.2 mL of 5% w/v sodium deoxycholate to the samples. Samples were incubated at 55°C for 90 min. Sample volumes were adjusted to 500 µL with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). An equal volume of phenol:chloroform:isoamyl acohol 25:24:1 was added before vortexing the sample for 30 s. After a 2 min spin at 16000 x g the top layer was transferred to a fresh 1.5 mL tube. The phenol-chloroform extraction was repeated 1-3 times. An equal volume of chloroform was added to the samples and they were vortexed for 30 s and spun for 2 min at 16000 x g. The top layer was transferred to a fresh 1.5 mL microcentrifuge tube. Ethanol precipitation was carried out by adding 0.1 vol of 3 M sodium acetate buffer (pH 5.2, chilled to 4°C) and 2.5 vol of 100% ethanol. Samples were incubated at -20°C overnight and subjected to a 15 min spin at 16000 x g. Supernatant was removed and the pellet was re-suspended in 1 mL of ice cold 100% ethanol. Samples were centrifuged for 2 min at 16000 x g and the supernatant was removed. The pellet was dried for 5-10 min at 30°C in an Eppendorf concentrator. Pellets were re-suspended in 50 µL TE buffer and DNA concentration was determined using a ND-1000 spectrophometer (NanoDrop®).

2.2.2 Extraction of mycobacterial DNA for colony PCR

One loopful of cells from a plate was added to 1 mL TE buffer. Samples were incubated for 10 min at 100°C. Samples were left to cool for 5 min and filter-sterilised through 0.2 µM filter units.

2.2.3 Extraction and purification of mycobacterial RNA

All RNA work undertaken outside of the ACDP Hazard Group 3 laboratory was carried out in a captair™ bio cabinet (erlab, Saisbury, UK) using RNase- and DNase-free filtered tips, water and microcentrifuge tubes. RNA was extracted as described by Rustad *et al.* (Rustad *et al.*, 2008b) *M. tuberculosis* cells from a 10 mL liquid culture were harvested by centrifugation for 10 min at 2700 x g. The pellet was resuspended in 1 mL TRI® Reagent (Invitrogen) and transferred to 2 mL tubes containing lysing matrix B. Cells were disrupted using two 30 s cycles at speed 6.5 using a FastPrep™ and placed on ice for 30 s. This step was repeated two more times. Samples were spun at 16000 x g for 4 min and filter-sterilised through a 0.2 µm filter unit.

The solution was removed to a 2 mL Heavy Phase Lock Gel (Eppendorf) snap cap tube containing 300 µL chloroform:isoamyl alcohol (24:1). Samples were mixed by inverting rapidly for 15 s and placed on ice. Once all samples were transferred, inverting was continued periodically for 2 min. Samples were spun for 5 min at 16000 x g and the aqueous layer (~540 µL) was removed to a 1.5 mL microcentrifuge tube containing 270 µL ispopropanol and 270 µL high salt solution (0.8 M sodium citrate, 1.2 M NaCl). Tubes were inverted several times to mix. Samples were left to precipitate overnight at 4°C and centrifuged for 10 min at 16000 x g at 4°C. The supernatant was removed and pellets were washed by adding 1 mL 75% ethanol. Tubes were inverted several times and spun for 5 min at 16000 x g before decanting the ethanol. Pellets were resuspended in 100 µL RNase-free water.

RNA was purified and DNase digested using the RNeasy® Minikit (QIAGEN) protocol. RLT buffer was prepared by adding 10 µL ß-mercaptoethanol per mL of buffer prior to use. 350 µL RLT buffer was added to the sample. The mixture was vortexed, 265 µL 95% ethanol was added and mixed by vortexing. The mixture was transferred to an RNeasy spin column, centrifuged for 15 s and transferred to a new 2 mL collection

tube. 350 µL of buffer RW1 was added and the column spun for 15 s before the flow through was discarded. 70 µL of buffer RDD was added to a 10 µL aliquot of DNase I stock solution and pipetted directly onto the column membrane. Digestions were allowed to continue at room temperature for 2-5 h. 350 µL buffer RW1 was added and the column spun for 15 s before adding 500 µL RPE buffer. The column was spun for 15 s and the flow through was discarded. An additional 500 µL RPE buffer was added and the column centrifuged for 2 min. Flow through was discarded and the column spun for 1 min to dry completely. The column was transferred to a new 1.5 mL tube and RNA eluted in 40 µL RNAse-free water. RNA concentrations were measured on a ND-1000 Spectrophometer. To check for RNA degradation, 1 µL of RNA sample was run on a 2% w/v agarose gel.

2.2.4 Preparation of complementary DNA (cDNA)

SuperScript™ II® reverse transcriptase (Invitrogen) was used for the preparation of cDNA according to manufacturer's instructions. Two tubes were prepared for each sample. In a total volume of 12 µL, 1-5 µg of DNA-free RNA was mixed with 1 µL Invitrogen random primers and 1 µL 10 mM deoxyribonucleotide triphosphates (dNTPs) (Promega). The mixture was incubated at 65°C for 5 min and chilled on ice. 4 µL of 5x First Strand Buffer, 2 µL 0.1 M dithiothreitol (DTT) and 1 µL RNasin® were added. After gentle mixing, the reaction was incubated at 25°C for 2 min. 1 µL of reverse transcriptase was added to one tube and 1 µL DNAse- and RNAse-free water was added to the negative control tube. Samples were mixed and incubated at 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. cDNA concentrations were measured on a ND-1000 spectrophometer.

2.3 Polymerase chain reaction (PCR)

2.3.1 PCR reactions using different polymerases

Reactions with GoTaq® Green Master Mix (Promega) (containing *Taq* DNA polymerase, buffer, $MgCl_2$ and dNTPs) were carried out in a total volume of 20 µL with 10-100 ng of DNA template, 1 µL of each 10 µM primer, 10 µL 2x Master Mix and SDW. Lists of primers used for this study are provided as appendices.

Reactions with Easy-A® high fidelity DNA polymerase (Stratagene) were carried out in a total volume of 50 µL containg 5 µL 10x buffer, 5 µL DMSO, 1 µL 10 mM dNTPs, 10-100 ng of DNA template, 2 µL of each 10 µM primer, 1 µL EasyA DNA polymerase and SDW.

Reactions with *Pfu* high fidelity DNA polymerase (Promega) were carried out in a total volume of 50 µL containing 5 µL 10x buffer, 1.5 µL DMSO, 1 µL 10 mM dNTPs, 10-100 ng of DNA template, 2.5 µL of each 10 µM primer, 1 µL *Pfu* DNA polymerase and SDW.

Reactions with Phusion™ Hot Start high fidelity DNA polymerase (Fynnzymes) were carried out in a total volume of 50 µL containing 10 µL 5x HF buffer, 5 µL DMSO, 1 µL 10 mM dNTPs, 10-100 ng of DNA template, 2.5 µL of each 10 µM primer, 0.38 µL Phusion DNA polymerase and SDW.

For semi-quantitative reverse transcription-PCR (RT-PCR), equal amounts of sample cDNA were diluted 1:4, 1:16, 1:64, 1:256 and 1:1024. In a total volume of 20 µL, 10 µL of 2x Master Mix, 2 µL DMSO, 1 µL of forward and reverse primer were mixed with 1 µL of template.

2.3.2 PCR amplification programmes

PCRs were carried out on a thermocycler TC-312 (Techne, Duxford, UK) or, for primer optimisation, on a Touchgene Gradient thermocycler (Techne) using the amplification programmes stated below.

For primer optimisation: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C (gradient 15°C) and extension at 72°C for 1 min. Final extension at 72°C for 5 min, followed by hold at 4°C.

For amplification of inserts: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at optimal temperature and extension at 72°C for 1 min. Final extension at 72°C for 5 min was followed by hold at 4°C.

For PCR reactions containing Phusion the following temperatures were used: initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at optimal temperature for 30 s and extension at 72°C for 30 s per 1 kb. Final extension at 72°C for 5 min was followed by hold at 4°C.

For semi-quantitative RT-PCR, the following program was used: Initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at optimal temperature and extension at 72°C for 1 min. Final extension at 72°C for 5 min was followed by hold at 4°C.

2.4 Preparation of DNA fragments for cloning, digests and gel electrophoresis

2.4.1 Cloning PCR products into pSC-A vector

A-tailed PCR products were cloned into pSC-A using the StrataClone™ PCR cloning kit (Stratagene). A reaction mixture was set up using 3 µL buffer, 10 ng PCR product and 1 µL Strataclone vector mix. After 5 min of incubation at room temperature, the reaction was placed on ice and transformed into StrataClone™ SoloPack® competent cells as follows: cells were thawed on ice, 1 µL of the reaction was added and mixed gently. Cells were incubated on ice for 20 min, subjected to a 45 s heat shock at 42°C, followed by 2 min on ice. Cells were recovered in 250 µL pre-warmed SOC medium shaking at 225 rpm at 37°C for 1 h. 100 µL and 5 µL of cells were plated onto LB agar plates containing the appropriate antibiotic and X-gal and incubated overnight. White or light blue colonies were picked.

2.4.2 Blunting of sticky-end DNA fragments

Sticky ends of DNA fragments were converted to blunt ends by mixing 1 µL *Pfu* high fidelity DNA polymerase (Promega), 2 µL of 10x buffer and 0.5 µL 10 mM dNTPs with 100-500 ng DNA and SDW in a 20 µL reaction. The mix was incubated for 25 min at 72°C. Samples were purified using a clean and concentrate kit (Zymo).

2.4.3 A-tailing of DNA fragments

DNA fragments were A-tailed in a total volume of 20 µL by mixing 4 µL of 5x buffer with 1 µL 100 mM deoxyadenosine triphosphates (dATPs) (Promega), 1 µL *GoTaq*® DNA polymerase (Promega) and 100-500 ng DNA. The mix was incubated for 30 min at 72°C. Samples were purified using a clean and concentrate kit (Zymo).

2.4.4 Dephosphorylation of DNA fragments

Sticky or blunt-end DNA was dephosphorylated using the Rapid DNA Dephos & Ligation kit (Roche) according to manufacturer's instructions.

The following mixture was prepared in a total volume of 20 µL: up to 1 µg of vector was added to 1 µL of rAPid Alkaline Phosphatase and 2 µL 10x buffer. The reaction was incubated at 37°C and assay time depended on the nature of the DNA end to be dephosphorylated. DNA with blunt or sticky 5' overhang ends: 10 min incubation. DNA with sticky 5' recessive ends: 30 min incubation. Dephosphorylation was followed by a 2 min inactivation at 75°C.

2.4.5 Restriction enzyme digests

For a 20 µL reaction, the digestion mix contained 200 ng of plasmid DNA, 2 µL of 10x enzyme buffer, 1-2 µL of enzyme and SDW. Digests were incubated at 37°C (except for *SmaI* which was incubated at 25°C) for 1-2 h.

For a 50 µL reaction, the digestion mix contained 1-5 µg of plasmid DNA, 5 µL of 10x enzyme buffer, 2-3 µL of enzyme and SDW. Where appropriate, 2 µL Alkaline Phosphatase (Promega) was added. Digests were incubated at 37°C (except for *SmaI* which was incubated at 25°C) overnight.

2.4.6 Gel electrophoresis and DNA extraction from agarose gels

Gel electrophoresis of DNA was performed on agarose gels made with UltraPure™ agarose (Invitrogen). Depending on DNA size, different percentage gels were made (0.8% w/v for large pieces of >5 kb or uncut DNA, 1.0% w/v for gel extractions or 1.5% w/v for small pieces of <3 kb) in 1x TAE (242 g L^{-1} Tris base, 37.2 g L^{-1} Na_2EDTA and 57.1 mL L^{-1} glacial acetic acid pH 8.3) with 0.5 µg mL^{-1} ethidium bromide. Orange G buffer (1x) was used as sample loading buffer (2.5 g L^{-1} Orange G sodium salt, 40% w/v glycerol, 60% w/v TE buffer).

To determine size of bands, a 1 kb DNA ladder (Promega) yielding bands of 10000, 8000, 6000, 5000, 4000, 3000, 2000, 1500, 1000, 750, 500 and 250 bp was used. 10 µL of a working stock of ladder (100 µL commercial ladder, 200 µL sample loading buffer and 200 µL SDW) was loaded on each agarose gel.

DNA was extracted from agarose gels using the QIAquick® Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. The desired band was excised using a scalpel and dissolved in 3 vol of buffer QG for 10 min at 50°C. To this, 1 gel vol of isopropanol was added and the mix was

applied to a QIAquick spin column. The DNA was bound to the column by centrifugation at 16000 x g for 1 min followed by a wash with 750 µL buffer PE and a centrifugation at 16000 x g for 1 min. DNA was eluted by applying 50 µL of SDW to the column and a centrifugation at 16000 x g for 1 min.

2.4.7 Cleaning and concentrating of DNA

Small quantities of DNA (≤5 µg) were cleaned after enzyme digests and concentrated using the "Zymo 5" DNA Clean and Concentrator-5™ kit (Zymo Research Corporation) according to manufacturer's instructions. Large quantities of DNA (between 5 µg and 25 µg) were cleaned and concentrated using the "Zymo 25" DNA Clean and Concentrator-25™ kit according to manufacturer's instructions. In a 1.5 mL microcentrifuge tube, 2 vol of DNA binding buffer was added to each vol of DNA sample and mixed briefly by vortexing. The mixture was transferred to a Zymo-Spin™ Column in a collection tube. The tubes were centrifuged at 16000 x g for 30 s and the flow-through discarded. 200 µL of wash buffer was added to the column before a centrifugation at 16000 x g for 30 s. Small quantities of DNA were eluted into a fresh 1.5 mL microcentrifuge tube in 6-10 µL of nuclease-free water by a 30 s centrifugation at 16000 x g. Large quantities of DNA were eluted into a fresh 1.5 mL microcentrifuge tube in 20-35 µL of nuclease-free water by a 30 s centrifugation at 16000 x g. DNA concentrations were measured on a ND-1000 spectrophometer.

2.4.8 Ligation of DNA fragments

Ligations were performed using the Rapid DNA Dephos & Ligation kit (Roche) according to manufacturer's instructions and with a vector:insert ratio of 1:3. Reactions were carried out in a total volume of 10 µL and contained 50 ng of linearised vector, a corresponding amount of insert, 2 µL of 5x dilution buffer and SDW. To this, 10 µL of 2x ligation buffer and 1 µL of DNA ligase were added. The ligation reaction was incubated at room temperature for 1 h before transformation into *E. coli*.

2.5 Transformation of *E. coli*, preparation of plasmid DNA and electroporation of mycobacteria

2.5.1 Transformation of *E. coli*

MAX Efficiency® DH5α™ competent cells (Invitrogen) or Library Efficiency® DH5α™ competent cells (Invitrogen) were used. Cells were thawed on ice.

1 µL of DNA (1-10 ng) was added to 100 µL of cells and mixed gently. Cells were incubated on ice for 30 min before being subjected to a 45 s heat shock at 42°C, followed by 2 min on ice. 0.9 mL of room temperature SOC medium (Invitrogen) was added and cells were incubated shaking at 225 rpm at 37°C for 1 h. 100 µL of cells and the pellet were plated onto an LB agar plate containing the appropriate antibiotic and grown overnight.

For subcloning, Subcloning Efficiency ™ competent cells (Invitrogen) were used. Cells were thawed on ice. 1-5 µL DNA (1-10 ng) was added to 50 µL of cells and mixed gently. Cells were incubated on ice for 30 min before being subjected to a 20 s heat shock at 42°C. This was followed by 2 min on ice. 950 µL of pre-warmed SOC medium was added and cells were incubated shaking at 225 rpm at 37°C for 1 h. 100 µL of cells and the pellet were plated onto an LB agar plate containing the appropriate antibiotic and grown overnight.

2.5.2 Preparation of plasmid DNA

Small scale plasmid DNA preparation i.e. Miniprep

Minipreps for small scale plasmid DNA preparations were carried out using the Wizard®*Plus* SV Minipreps DNA Purification Systems kit (Promega) according to manufacturer's instructions. A 10 mL culture was grown overnight and harvested by centrifugation (5 min at 2700 x g). The pellet was resuspended in 250 µL cell resuspension solution, 250 µL of cell lysis solution was added and mixed by inversion, 10 µL of alkaline protease was added and incubated at room temperature for 5 min, 350 µL of neutralisation solution was added and mixed by inversion. The mix was centrifuged at 16000 x g for 10 min. The cleared lysate was decanted into a spin column and spun for 1 min at 16000 x g, 750 µL wash solution was added to the column and spun for 1 min at 16000 x g. The wash step was repeated with 250 µL wash solution followed by a 2 min spin. DNA was eluted by adding 50 µL nuclease-free water to the column and centrifugation for 1 min at 16000 x g. DNA was stored at -20°C.

Large scale plasmid DNA preparation i.e. Midiprep

Midipreps for large scale plasmid DNA preparations were carried out using the HiSpeed Plasmid purification kit (QIAGEN) according to manufacturer's instructions. A 100 mL culture was grown overnight and harvested by

centrifugation (10 min at 2700 x g). The pellet was resuspended in 6 mL of buffer P1, 6 mL buffer P2 was added, mixed by inversion and incubated at room temperature for 5 min; 6 mL of chilled buffer P3 was added and mixed by inversion. The lysate was poured into a QIAfilter cartridge and incubated for 10 min at RT. The cleared lysate was transferred into a HiSpeed tip (pre-equilibriated with 4 mL buffer QBT) and allowed to enter the resin by gravity flow. The HiSpeed tip was washed with 20 mL buffer QC, DNA was eluted with 5 mL of buffer Q, precipitated by the addition of 3.5 mL isopropanol and incubated at room temperature for 5 min. The solution was passed through a QIAprecipitor, washed with 2 mL of 70% ethanol and DNA was eluted in 1 mL TE buffer and stored at -20°C. DNA concentrations were measured on a ND-1000 spectrophometer.

Lists of plasmids used for this study are provided as appendices.

2.5.3 Site directed mutagenesis (SDM)

PCR amplification reactions were carried out in 50 µL total volume containing 2.5 units *PfuUltra*® Hot Start high fidelity DNA polymerase (Stratagene), 1x buffer, 0.5 mM dNTPs, 10 pmol of primer, 5 µL DMSO, and 10 ng template. The thermocycling programme used was: 94°C for 2 min, followed by 18 cycles of 94°C for 30 s, 56°C for 1 min and 68°C for 9 min, followed by 68°C for 10min. Template was degraded using 10 units *DpnI* (Promega) at 37° for 2 h. 10 µL of each reaction were used to transform competent *E. coli*. Recombinant plasmids were isolated and sequence-verified.

2.5.4 Construction of plasmids for testing promoter activity

For each of the genes selected in this study, a region of 250–600 bp upstream of the predicted translational start site was PCR-amplified using the primers listed in the appendices. *SmaI* restriction sites were incorporated into the primers where required. The amplified regions were cloned into the PCR cloning vector pSC-A from which they were excised using *SmaI*. Inserts were cloned into the *ScaI* site of the mycobacteriophage L5-derived promoter probe vector pSM128 unless otherwise stated. Successful cloning was confirmed by restriction enzyme digest and sequence verification. Lists of plasmids used in this study are provided as appendix. pSM128 is an integrative vector that contains the promoterless reporter gene *lacZ*, which encodes the enzyme ß-

galactosidase (Dussurget *et al.,* 1999). Insertion of a promoter region upstream of *lacZ* would result in expression of *lacZ*. ß-galactosidase activity was assessed by measuring cleavage of ONPG and is given as a standardised amount in Miller Units.

Upstream regions of genes that have been shown to be induced by streptomycin (the antibiotc selection marker of pSM128) were cloned into pFLAME3 and pFLAME4, which use kanamycin as selective marker. After sequence verification, inserts were excised from pSC-A using *EcoRI* and cloned into the *EcoR*I site of the vectors pFLAME3 and pFLAME4. Successful cloning was confirmed by restriction enzyme digest and sequence verification. The replicating vectors pFLAME3 and pFLAME4 contain the promoterless reporter gene *gfp* (Blokpoel *et al.,* 2003).

2.5.5 Construction of promoter probe vectors containing unstable LacZ

Protein tags (AADENYA-ASV and AADENYA-LAA) were added to the *lacZ* in pSM128 in two rounds of SDM using LAA, ASV, tail.LAA and tail.ASV primers (see appendix). PCR-amplified upstream regions were cut out of pSC-A and cloned into the *SmaI* sites of the plasmids containing the tagged LacZ. Successful cloning was confirmed by restriction enzyme digest and sequence verification.

2.5.6 Construction of antisense plasmids

LacZ knockdown plasmids were constructed as follows: P_{27} was cut out of pDS53 using *KpnI* and cloned into the *KpnI* site of the replicative vector pMV206 to create pDS171. Successful cloning was confirmed by restriction enzyme digests. Primers were designed to amplify *lacZ* from pSM128. Primers contained a *HindIII* and a *BamHI* site for directional cloning of *lacZ* in an antisense direction directly downstream of P_{27} into pDS171. Successful cloning was verified by restriction enzyme digests.

P_{sal} was cut out of pDS167 using *KpnI* and cloned into the *KpnI* site of the replicative vector pMV206 to create pDS193. Successful cloning was confirmed by restriction enzyme digests. Primers were designed to amplify *lacZ* from pSM128. Primers contained a *HindIII* and a *BamHI* site for directional cloning of *lacZ* in an antisense direction directly downstream of

P_{sal} into pDS193. Successful cloning was verified by restriction enzyme digests.

RpoB knockdown plasmids were constructed as follows: Primers were designed to amplify full length *rpoB* from the mycobacterial genome. Primers contained a *HindIII* and a *BamHI* site for directional cloning of *rpoB* in an antisense direction to the promoters in in pDS171 or pDS193. Successful cloning was verified by restriction enzyme digests.

2.5.7 Electroporation of mycobacteria

Electroporation of *M. smegmatis*

A 5 mL pre-culture was grown overnight and 1 mL was used to inoculate a 100 mL culture, which was grown for 18-24 h until the OD_{600} reached 0.8-1.0 The culture was chilled on ice for 90 min before pelleting the cells by centrifugation at 2700 x g for 10 min at 4°C. The pellet was resuspended in 20 mL 10% w/v glycerol, washed in 5 mL 10% w/v glycerol and resuspended in 1 mL 10% w/v glycerol. 200 µL aliquots were dispensed for storage at -80°C. Electrocompetent cells were thawed on ice. To each vial of cells, 1 µg of plasmid DNA (in a volume of ≤5 µL) was added, mixed gently and the sample incubated on ice for 10 min before transferral into a pre-chilled 2 mm electroporation cuvette (Flowgen Biosciences). Cells were pulsed in an electroporator (Bio-Rad Laboratories Ltd.) set at 2.5 kV, 25 µF and 1000 Ω. Cells were incubated on ice for 10 min, recovered in 5 mL Lemco broth and grown at 37°C for 2 h with shaking at 100 rpm. Serial dilutions were plated onto Lemco agar plates containing a selective antibiotic. Transformants were picked after 3-5 d of incubation at 37°C.

Electroporation of *M. tuberculosis*

An inoculum of 3 mL was used to set up a 100 mL roller culture, which was grown for 7 d. 24 h before harvesting, the culture was treated with 10 mL of 2 M glycine. 50 mL of culture was pelleted by centrifugation at 2700 x g for 10 min, washed twice in pre-warmed 10% w/v glycerol (10 and 5 mL respectively) and resuspended in 1.2 mL 10% w/v glycerol. 1 µg of plasmid DNA (in a volume of ≤5 µL) was mixed with 200 µL of cells before transferral into a 2 mm electroporation cuvette. Cells were pulsed in an electroporator set to 2.5 kV, 25 µF and 1000 Ω. Cells were recovered in 10 mL medium and incubated at 37°C overnight. Serial dilutions were plated

onto solid medium containing the appropriate antibiotic. Transformants were picked after 3 weeks of growth.

2.6 Reporter gene assays

2.6.1 Preparation of cell-free extracts

M. smegmatis cells were harvested by centrifugation (10 min at 2700 x g), washed in 5 mL 10 mM Tris-Cl (pH 8), resuspended in 1 mL 10mM Tris-Cl (pH 8) and added to 2 mL lysing matrix B tubes (MP Biomedicals) on ice. Cells were disrupted using two 30 s cycles at speed 6.0 using a FastPrep^{TM} FP120 and incubated for 5 min on ice between cycles. Samples were spun at 16000 x g for 1 min and the supernatants were recovered. Cell-free extracts from *M. tuberculosis* cells were prepared using the protocol above except that the cells were disrupted once and spun at 16000 x g for 4 min, and filter-sterilised through a 0.2 µm filter unit.

2.6.2 Determination of protein concentration in cell-free extracts

Total protein concentration of the samples was determined using the Pierce BCA protein assay kit (Fisher). Standards of bovine serum albumin (BSA) were prepared using 0.9% w/v NaCl and used at 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg mL^{-1} or at 0, 25, 50, 75, 100, 150, 200, 300, 400 and 500 µg mL^{-1}. Reagents A and B were mixed at a ratio of 50:1 and 200 µL of this solution was added to 25 µL of standard or cell-free extract in a 96-well PVC microtitre plate. Samples were incubated at 37°C for 30 min and the OD_{562} was measured. A standard curve was plotted from which protein concentrations of the cell-free extracts were calculated.

2.6.3 ß-galactosidase activity assay

Assays of ß-galactosidase activity were carried out as previously described (Miller, 1972). To 100 µL of cell-free extract, 900 µL of Z-Buffer (60 mM Na_2HPO_4, 40 mM Na_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7) was added. Samples were pre-warmed to 37°C for 5 min and 200 µL of 4 mg mL^{-1} ONPG was added. Reaction mixtures were incubated at 37°C and reactions were stopped with 500 µL of 1 M $NaHCO_3$ after 30, 45, 60 or 90 min. The OD_{420} was measured and ß-galactosidase activity was calculated as Miller units (MU) using the formula:

$$\text{Units} = \frac{OD_{420} \times 1.7}{\text{time (min)} \times \text{volume of cell-free extract (mL)} \times \text{total protein concentration (mg mL}^{-1}) \times 0.0045}$$

2.6.4 High sensitivity ß-galactosidase activity assay

For promoter activites under 40 MU, a High Sensitivity ß-galactosidase Activity Assay kit (Stratagene) was used according to manufacturer's instructions. 100 µL of cell lysate was transferred to a 1.5 mL microcentrifuge tube. 900 µL of 1x chlorophenol red ß-D-galactopyranoside (CPRG) substrate was added to the samples and vortexed to mix. Reaction mixtures were kept at 37°C for 30 min or longer (up to 72 h) in a water bath until the samples turned dark red. The reactions were stopped by adding 500 µL stop solution and the optical density of the experimental and the blank (containing 100 µL of 10 mM Tris pH 8) was measured at a wavelength of 570-595 nm. Specific ß-galactosidase activity (units mg^{-1}) was determined by the following formula:

$$\text{Specific ß-galactosidase activity} = \frac{OD_{580} \times 55}{\text{time (min)} \times \text{volume of cell-free extract (mL)} \times \text{total protein concentration (mg mL}^{-1})}$$

Activity of ß-galactosidase was expressed as nmol of chlorophenol red formed/min/mg of total protein which equals U of ß-galactosidase activity/mg of total protein.

2.6.5 Assaying fluorescence

When using the reporter gene *gfp*, promoter activity was assayed by determining the fluorescence intensity in cell-free extracts. 100 µL of cell-free extract was added to a black 96-well plate (Nalge NUNC) and fluorescence intensity was read at an excitation of 483 nm and an emission of 520 nm on a FLUOstar OPTIMA fluorimeter (BMG LABTECH Ltd.). Relative fluorescence was determined by dividing the fluorescence intensity by the protein concentration of the samples as determined by BCA protein assay. Fluorescence was expressed in relative fluorescence units (RFU) - fluorescence intensity per mg of protein.

2.7 Statistical analysis

The Student's T test (unpaired, two sided) was used for statistical analysis of data generated in this study. P-values of <0.05 were considered

statistically significant at a 95 % confidence interval. Statistical analysis was performed using Microsoft Office Excel software.

For comparison of expression profiles, analysis of covariance (ANCOVA) was performed to test if slopes of two linear regression lines were different. P-values of <0.05 were considered statistically significant at a 95 % confidence interval. Statistical analysis was performed using Minitab®15 statistical software.

3. Identification of endogenous antibiotic-inducible promoters

3.1 Introduction

Inducible promoter systems can be used to control expression of a target gene under various conditions. Thus they can be utilised to elucidate the function of a gene under a condition of interest or be employed to prove gene essentiality for growth *in vitro* or *in vivo*. Any gene essential for mycobacterial survival is a potential drug target. Inducible, titratable expression systems can be employed to determine target vulnerability as well as to confirm target specificity of a drug developed for a particular target. One of the main advantages of having a system regulated by an antibiotic is that it can be used to control an inducible promoter system in an *in vivo* setting, since many antibiotics such as tetracycline are able to enter mammalian cells, as well as penetrate mycobacterial cell walls (Bocker *et al.*, 1981). Tetracycline-inducible systems are amongst the most popular to date and have been used for the study of gene function and construction of conditional mutants in various bacteria, including mycobacteria (Berens and Hillen 2003; Chalut *et al.,* 2006; Corbel and Rossi 2002; Ehrt and Schnappinger 2006; Gandotra *et al.,* 2007; Geissendorfer and Hillen 1990; Guo *et al.,* 2007; Ji *et al.,* 1999; Lutz and Bujard 1997; Qian and Pan 2002; Tauch *et al.,* 2000).

3.1.1 Antibiotic-inducible promoter systems for use in mycobacteria

In mycobacteria, the most successful and widely-used inducible systems are regulated by an antibiotic, either tetracycline, its derivative anhydrotetracycline or pristinamycin (Blokpoel *et al,.* 2005; Carroll *et al,.* 2005; Ehrt *et al.,* 2005; Forti *et al.,* 2009; Gandotra *et al.,* 2007; Guo *et al.,* 2007; Hernandez-Abanto *et al.,* 2006; Klotzsche *et al.,* 2009; Rao *et al,.* 2008). Their advantage lies in the fact that they are genetically stable and can be successfully used in both fast- and, more importantly, slow-growing mycobacteria. Furthermore, the tetracycline- and anhydrotetracycline-inducible systems have been shown to be functional in both *in vitro* and *in vivo* studies (Blokpoel *et al,.* 2005; Carroll *et al,.* 2005; Ehrt *et al.,* 2005; Forti *et al.,* 2009; Gandotra *et al.,* 2007; Guo *et al.,* 2007; Hernandez-Abanto *et al.,* 2006; Klotzsche *et al.,* 2009; Rao *et al,.* 2008). Despite these

obvious advantages, the tetracycline-inducible systems have suffered from limitations such as a lack of tight control and high background activity (Blokpoel *et al,.* 2005; Carroll *et al.,* 2005; Ehrt *et al.,* 2005).

3.1.2 Antibiotic-inducible genes in *M. tuberculosis*

The use of an antibiotic-inducible promoter native to *M. tuberculosis* might ensure a tighter regulation and genetic stability, whilst retaining the obvious advantage of being suitable for *in vivo* use, since antibiotics can be administered to animals during an infection model. There are several reports of antibiotic-inducible genes in *M. tuberculosis*, i.e. *iniA* is induced by isoniazid and ethambutol (Colangeli *et al.,* 2005), and the *whiB*-like genes are induced by cycloserine, ethambutol, isoniazid, streptomycin and kanamycin (Geiman *et al.*, 2006). The WhiB family of transcriptional regulators which is common to *Streptomyces*, *Mycobacteria* and *Nocardia* species (Soliveri *et al.,* 2000). *M. tuberculosis* contains seven *whiB*-like genes, *whiB1-7*, which are structurally and functionally divergent (Alam *et al.*, 2009). Whilst the exact function of most of these is unknown, *whiB*3 is a redox sensor essential for a metabolic switch for survival of nutrient starvation; *whiB7* regulates expression of genes involved in ribosomal protection and antibiotic efflux (Morris *et al.,* 2005).

A large microarray study characterising *M. tuberculosis* gene expression under a plethora of conditions showed a number of *M. tuberculosis* genes to be upregulated after treatment with a variety of antibiotics including amikacin, capreomycin, roxithromycin, streptomycin and tetracycline (Boshoff *et al.,* 2004).

The aim of this project was to develop new inducible promoters for use as expression systems. This was to be achieved by identifying and characterising a wide range of endogenous inducible promoters of *M. tuberculosis* and ultimately using these to express genes of interest in a controllable fashion under a variety of conditions, including *in vivo* infection models.

3.2 Results

3.2.1 Selection of candidate genes

To ensure that an antibiotic-inducible system would work in *M. tuberculosis*, it was decided to look for endogenous genes that are highly upregulated in

response to antibiotics and to test their upstream regions for promoter activity by linking them to a promoterless reporter protein. This would establish if differential expression in response to antibiotic occured and enable the identification of antibiotic-inducible promoters.

Gene expression data (Boshoff *et al.,* 2004) was mined to find clusters of genes that were most upregulated in response to tetracycline. Thirty six clusters were found to be induced at least 10-fold after tetracycline treatment. To avoid unwanted induction or repression of the system under other conditions of interest, clusters were sorted according to whether they were expressed under conditions such as nutrient starvation, hypoxia, growth with succinate as sole carbon source, low pH or exposure to H_2O_2. Clusters that were upregulated or downregulated under these conditions were excluded from the selection. From the remaining nine clusters (containing a total of 136 genes), genes that were the most responsive to tetracycline (with an upregulation of ~70-fold) were selected. To avoid the problem of background activity in the absence of inducer, a further criteria for selection was introduced: only genes that were not or weakly expressed during aerobic exponential phase growth were selected for further study. The following genes were chosen to be tested for promoter inducibility in response to tetracycline: Rv0277c, Rv0609, Rv0749, Rv1015c (*rplY*), Rv2487c and Rv3898c.

Rv0277c, Rv0749 and Rv0609 are non-essential conserved hypothetical proteins (CHPs) that are part of toxin-antitoxin (TA) systems (Sassetti *et al.,* 2003; Arcus *et al.,* 2005). Due to the fact that both the antitoxin and the toxin of TA systems are expressed on bicistronic mRNAs from the promoter of the antitoxin, the upstream region the antitoxin was tested for inducibilty (thus Rv0608 as opposed to Rv0609 and Rv0748 as opposed to Rv0749 were chosen) (Suzuki *et al.*, 2005). The other three genes selected, Rv1015c, Rv2487c and Rv3898c are also predicted to be non-essential (Lamichhane *et al.,* 2003; Sassetti *et al.,* 2003). Rv2487c is a member of the PE PGRS family (PE PGRS42) of unknown function; Rv3898c is a hypothetical protein of unknown function; and Rv1015c (*rplY*) is a probable 50S ribosomal protein (TubercuList).

In addition to the tetracycline-responsive genes described above, members of the antibiotic-inducible WhiB family were also selected for further study.

WhiB7 (streptomycin and kanamycin-inducible) and *whiB6* (ethanol and SDS-inducible) were selected from RT-PCR data (Geiman *et al.*, 2006). *WhiB6* and *whiB7* are induced during late stationary phase growth. However, *whiB*7 was chosen because it showed the highest fold induction in response to streptomycin (70-fold) and kanamycin (over 70-fold). *WhiB*6 was also chosen to be tested since it showed the highest induction in response to treatment with SDS or ethanol (15-fold and 4-fold respectively) (Geiman *et al.*, 2006).

3.2.2 Testing for inducible promoter activity in *M. tuberculosis*

None of the promoters of these genes have been characterised, nor have their translational start sites been confirmed. To determine if the upstream regions of the selected genes contained promoters that were activated in response to an inducing stimulus, promoter probe plasmids were constructed by linking the upstream region of each of the selected genes to a promoterless reporter gene. To ensure inclusion of the promoter and any regulatory region(s), 200-550 bp upstream of the predicted translational start and a portion of the ORF was tested for promoter activity (Fig 3.1 and 3.2).

Each upstream region was cloned into the promoter probe vectors pSM128 or pFLAME3 and pFLAME4. The vector pSM128 is an integrative plasmid that contains *lacZ* reporter gene encoding the enzyme ß-galactosidase (Dussurget *et al.,* 1999). Insertion of a promoter region upstream of *lacZ* would result in expression of *lacZ*. The upstream regions of Rv0277c, Rv1015c and Rv2487c were cloned into pSM128. The upstream regions of Rv0608, Rv0748, Rv3898c, *whiB6* and *whiB7* were cloned into pFLAME3 and pFLAME4 because they are potentially responsive to streptomycin, the antibiotic selection marker of pSM128. The vectors pFLAME3 and pFLAME4 are episomal multicopy plasmids, containing the promoterless reporter gene *gfp* (Blokpoel *et al.*, 2003). The GFP in these vectors has been rendered unstable by the addition of a protein tag, AANDENYAASV in pFLAME3 and AANDENYALAA in pFLAME4 which targets the protein for ssRA (small stable RNA A) mediated degradation (Blokpoel *et al.*, 2003; Karzai *et al.*, 2000; Keiler *et al.*, 1996; Tyagi and Kinger, 1992). The two

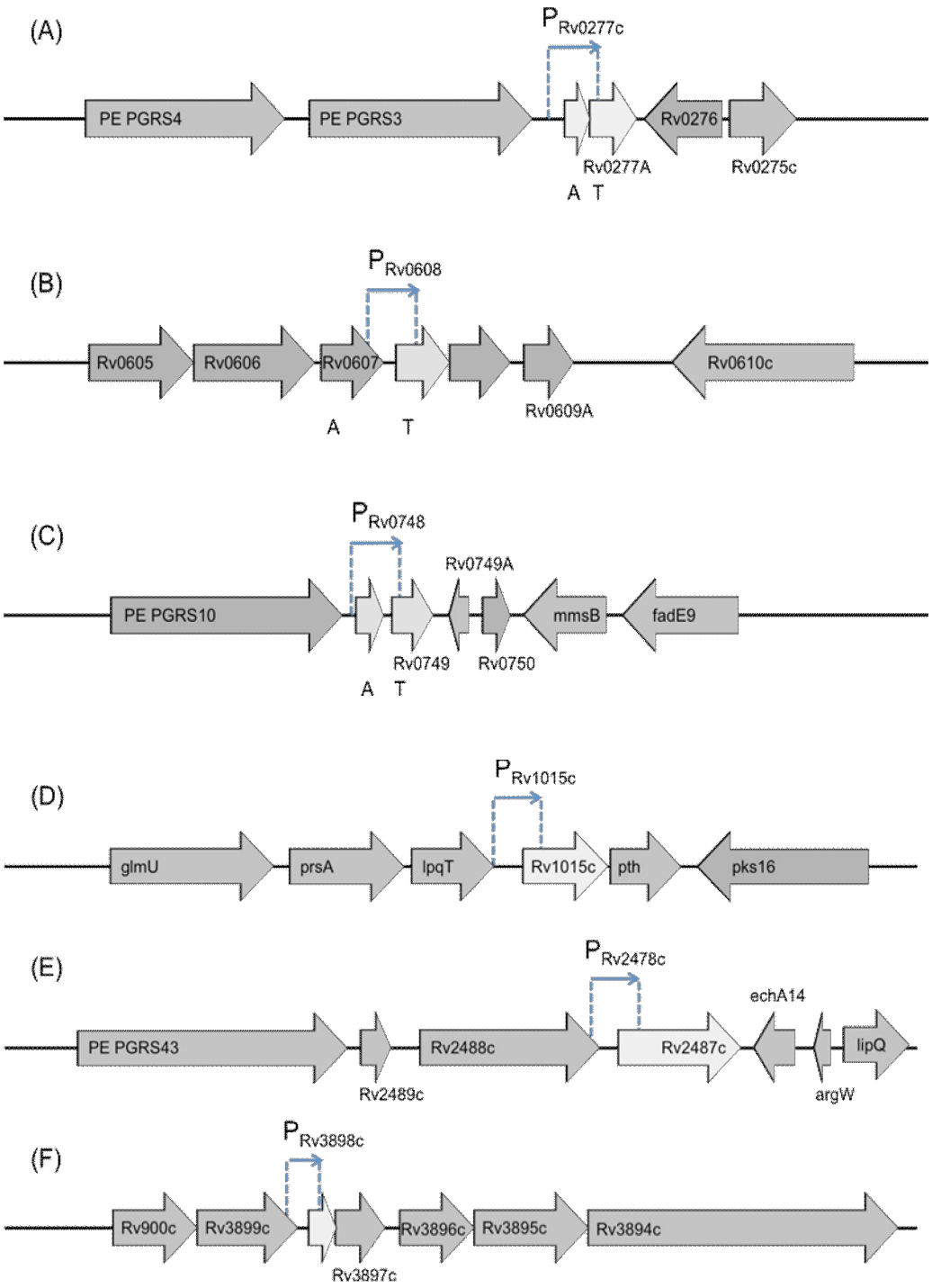

Figure 3.1. Genetic organisation of tetracycline-inducible genes selected for analysis.

Lightly shaded arrows represent the open reading frames of (A) Rv0277c, (B) Rv0608, (C) Rv0748, (D) Rv1015c, (E) Rv2487c and (F) Rv3898c. Adjacent open reading frames are denoted by arrows in dark grey. For genes part of a TA system, the antitoxin is marked by an “A” and the toxin by a “T” underneath the ORF.

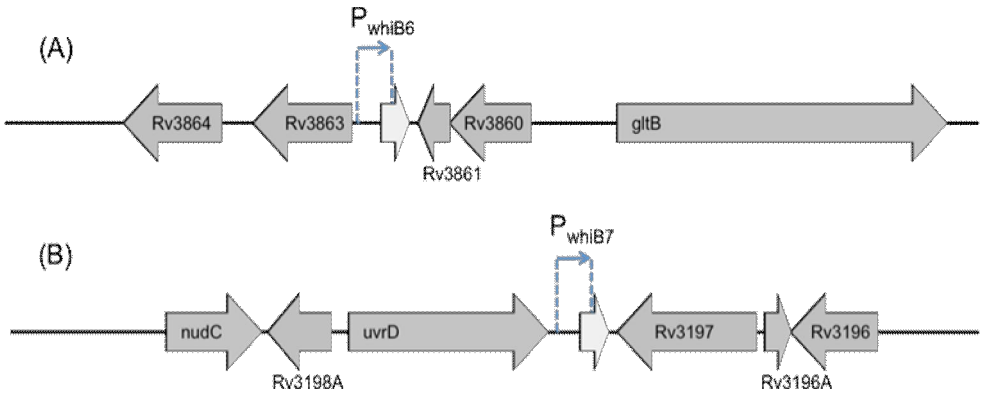

Figure 3.2. Genetic organisation of WhiB family genes selected for analysis.

Lightly shaded arrows represent the open reading frames of (A) *whiB6* and (B) *whiB7*. Adjacent open reading frames are denoted by arrows in dark grey.

tags differ in the resulting half-life of GFP produced from pFLAME3 and pFLAME4 (Blokpoel *et al.,* 2003).

To probe that the upstream regions of these genes contained promoters that were indeed inducible, plasmids were electroporated into *M. tuberculosis* and promoter activity was assayed.
Liquid cultures of *M. tuberculosis* transformants carrying each plasmid were subjected to treatment with inducing stimuli: tetracycline for $P_{Rv0277c}$, P_{Rv0608}, P_{Rv0748}, $P_{Rv1015c}$, $P_{Rv2487c}$, $P_{Rv3898c}$, streptomycin for P_{whiB7} and ethanol or SDS for P_{whiB6} (Tables 3.1 and 3.2).

For upstream regions $P_{Rv0277c}$ and $P_{Rv1015c}$, no promoter activity was detected before or after treatment with tetracycline (Table 3.1). Very weak promoter activity was detected from $P_{Rv2487c}$ in the absence of a stimulus ($p < 0.05$) (Table 3.1). Promoter activity was induced 2-fold after tetracycline exposure.

Very weak, non-inducible promoter activity was measured for P_{Rv0608} ($p < 0.05$), but only when linked to GFP-LAA (Table 3.2). No promoter activity was detected in the upstream region P_{Rv0608} when linked to GFP-ASV. For upstream regions P_{Rv0748} and $P_{Rv3898c}$, no promoter activity was detected under aerobic growth conditions or after exposure to tetracycline with either tagged versions of GFP (Table 3.2). In the absence of inducer, no promoter activity was detected from P_{whiB7} regardless of the type of GFP reporter used (Table 3.2). Promoter activity was not induced by streptomycin. For upstream region P_{whiB6}, promoter activity was observed under non-inducing conditions ($p < 0.05$) (Table 3.2). However, the promoter was not induced in response to treatment with either ethanol or SDS.

Except for $P_{Rv2487c}$, P_{Rv0608} and P_{whiB6}, none of the upstream regions tested were found to have promoter activity significantly different from the background in the absence of a stimulus. This was as expected as one of the selection criteria was no or low expression during normal aerobic growth. Surprisingly, none of the upstream regions, apart from $P_{Rv2487c}$, were found to contain inducible promoter activity by the stimuli described in the literature (Boshoff *et al.*, 2004; Geiman *et al.*, 2006).

Upstream region	Activity (MU) -	Activity (MU) +
$P_{Rv0277c}$	8 ± 4	8 ± 1
$P_{Rv1015c}$	9 ± 3	6 ± 1
$P_{Rv2487c}$	7 ± 2	15 ± 6*

Table 3.1. Promoter activity of $P_{Rv0277c}$, $P_{Rv1015c}$ and $P_{Rv2478c}$ in *M. tuberculosis*.

ß-galactosidase activity of upstream regions of potentially antibiotic-responsive genes linked to promoterless *lacZ* was measured during exponential growth (uninduced) and after exposure to 10 µg mL^{-1} tetracycline for 6 h (induced). The average and standard deviation of three independent transformants assayed in duplicate is given. ß-galactosidase activity is given in Miller Units (MU) - measured as nmol of O-nitrophenol produced over time (min) per mg of protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the uninduced control is marked by an * for $p < 0.05$.

Upstream region	Reporter	Stimulus	Fluorescence (RFU) -	Fluorescence (RFU) +
P_{Rv0608}	GFP-ASV	Tetracycline	9 ± 1	9 ± 1
	GFP-LAA	Tetracycline	13 ± 1	14 ± 2
P_{Rv0748}	GFP-ASV	Tetracycline	7 ± 1	6 ± 1
	GFP-LAA	Tetracycline	8 ± 1	9 ± 1
$P_{Rv3898c}$	GFP-ASV	Tetracycline	7 ± 2	7 ± 1
	GFP-LAA	Tetracycline	8 ± 1	9 ± 1
P_{whiB7}	GFP-ASV	Streptomycin	5 ± 1	5 ± 1
	GFP-LAA	Streptomycin	5 ± 1	6 ± 1
P_{whiB6}	GFP-ASV	Ethanol	15 ± 5	12 ± 1
	GFP-LAA	Ethanol	19 ± 5	21 ± 2
	GFP-ASV	SDS	15 ± 5	15 ± 2
	GFP-LAA	SDS	19 ± 5	18 ± 1

Table 3.2. Promoter activity of P_{Rv0608}, P_{Rv0748}, $P_{Rv3898c}$, P_{whiB7} and P_{whiB6} in *M. tuberculosis*.

Fluorescence of upstream regions of antibiotic-, ethanol- and SDS-responsive genes linked to two unstable versions of promoterless *gfp* during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to tetracycline (10 μg mL^{-1} for 6 h), streptomycin (7.5 μg mL^{-1} for 3.5 h), ethanol (2.5% for 1.5 h) or SDS (0.05% for 1.5 h). The average ± standard deviation of three independent transformants assayed in duplicate is given in Relative Fluorescence Units (RFU) - measured as fluorescence intensity per mg of protein in cell-free extracts. The background fluoresence from empty vectors pFLAME3 (carrying GFP-ASV) and pFLAME4 (GFP-LAA) was 7 ± 2 RFU and 7 ± 3 RFU respectively.

Testing for promoter activity in *M. tuberculosis* in response to stimulation with lower concentrations of inducer

No induction of promoter activity was seen in response to treatments with tetracycline or streptomycin for all but one of the upstream region tested. Both of the antibiotics used as inducers interfere with translation (Alberts, 2002). Streptomycin is a bacteriocidal antibiotic which binds to the S12 protein of the 30S ribosomal subunit and inhibits protein synthesis. Tetracycline is a bacteriostatic antibiotic which inhibits protein synthesis by preventing binding of aminoacyl transfer RNA to the ribosome A site (Alberts, 2002). It is possible that the concentration and/or length of treatment used inhibited protein synthesis, thus even if promoter activity had been induced this would only have been observed at the level of transcription and not translation. In order to rule out this possibility, it was decided to test for induction using smaller amounts of drug.

Promoter activity was measured in *M. tuberculosis* before and after treatment with either 50 ng mL^{-1} tetracycline ($P_{Rv0277c}$, P_{Rv0608}, P_{Rv0748}, $P_{Rv1015c}$, $P_{Rv2487c}$, $P_{Rv3898c}$) or 50 ng mL^{-1} streptomycin (P_{whiB7}) (Tables 3.3 and 3.4).

For upstream regions $P_{Rv0277c}$ and $P_{Rv2487c}$, no promoter activity was detected in the absence or presence of a stimulus, since ß-galactosidase activity was in the same range as that of the empty vector control (Table 3.3). The fact that no promoter activity was detected from $P_{Rv2487c}$ under non-inducing conditions implies that activity observed in the previous experiment may have been an anomaly. Very weak promoter activity was detected from $P_{Rv1015c}$ ($p < 0.05$), albeit only in the absence of tetracycline.

For upstream regions P_{Rv0608}, P_{Rv0748}, $P_{Rv3898c}$ and P_{whiB7}, only one transformant was assayed and fluorescence was within the range of the empty vectors regardless of the presence or absence of a stimulus or type of reporter used (Table 3.4).

Overall, promoter activities were not induced when using a lower concentration of tetracycline or streptomycin.

The previous data showed that lowering the concentration of tetracycline or streptomycin used for stimulation by several 100-fold did not result in induction of promoter activity. It is possible that the dynamics of induction

Upstream region	Activity (MU) -	Activity (MU) +
$P_{Rv0277c}$	10 ± 1	6 ± 1*
$P_{Rv1015c}$	11 ± 2	13 ± 3
$P_{Rv2487c}$	9 ± 2	5 ± 1*

Table 3.3. Promoter activity of $P_{Rv0277c}$, $P_{Rv1015c}$ and $P_{Rv2487c}$ in *M. tuberculosis*.

ß-galactosidase activity of upstream regions of potentially antibiotic-responsive genes linked to promoterless *lacZ* during exponential growth (-) and after exposure (+) to 50 ng mL^{-1} tetracycline for 6 h is shown. The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (MU) - measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the uninduced control is marked by an * for $p < 0.05$.

Upstream region	Reporter	Stimulus	Fluorescence (RFU) -	Fluorescence (RFU) +
P_{Rv0608}	GFP-ASV	Tetracycline	6	4
	GFP-LAA	Tetracycline	6	7
P_{Rv0748}	GFP-ASV	Tetracycline	3	6
	GFP-LAA	Tetracycline	6	7
$P_{Rv3898c}$	GFP-ASV	Tetracycline	4	3
	GFP-LAA	Tetracycline	6	4
P_{whiB7}	GFP-ASV	Streptomycin	4	2
	GFP-LAA	Streptomycin	3	3

Table 3.4. Promoter activity of P_{Rv0608}, P_{Rv0748}, $P_{Rv3898c}$ and P_{whiB7} in *M. tuberculosis*.

Fluorescence of upstream regions of potentially antibiotic-responsive genes linked to two unstable versions of promoterless *gfp* during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to tetracycline (50 ng mL^{-1} for 6 h) or streptomycin (50 ng mL^{-1} for 3.5 h). The fluorescence of one transformant assayed in duplicate is given in Relative Fluorescence Units (RFU) - measured as fluorescence intensity per mg of protein in cell-free extracts. The background fluorescence from empty vectors pFLAME3 (carrying GFP-ASV) and pFLAME4 (GFP-LAA) was 7 RFU and 8 RFU respectively.

might have changed at the lower concentrations i.e. using less inducer would require exposure for a longer time before an effect could be observed. Therefore it was decided to increase treatment time to 24 h.

Promoter activity was measured in cultures subjected to treatment with either 50 ng mL^{-1} tetracycline ($P_{Rv0277c}$, P_{Rv0608}, P_{Rv0748}, $P_{Rv1015c}$, $P_{Rv2487c}$, $P_{Rv3898c}$,) or 50 ng mL^{-1} streptomycin (P_{whiB7}).

For upstream regions $P_{Rv1015c}$, $P_{Rv0277c}$ and $P_{Rv2487c}$, no promoter activity was detected in the absence or presence of a stimulus (Table 3.5).

The same was observed for P_{Rv0608}, P_{Rv0748}, $P_{Rv3898c}$ and P_{whiB7}, where fluorescence remained at the level of the vector control even with the increased treatment length (Table 3.6).

None of the upstream regions tested were found to have promoter activity that differed significantly from the background and they were not found to be induced even when less antibiotic was being used and cultures were treated for longer.

Activity of constitutively active promoters in *M. tuberculosis* in response to antibiotic treatment

Lack of induction of promoter activity in the upstream regions tested may have been due to the treatments inhibiting protein synthesis, even at the lower concentrations. An alternative explanation for lack of induction could be that there were no promoters in the upstream regions. In order to test whether lack of induction was due to a block of translation, it was decided to test activity of constitutively active promoters linked to all three types of reporter genes after exposure. If the antibiotic treatments chosen interfered with protein synthesis, then promoter activity in the samples would be reduced under these conditions.

The following constructs of constitutively active promoters linked to reporter genes were used: $P_{Antigen\ 85A}$ with *lacZ* (pDS165), $P_{myc1tetO}$ with GFP-ASV (pHLEGM7) and $P_{myc1tetO}$ with GFP-LAA (pHLEGM8).

Plasmids pDS165, pHLEGM7 and pHLEGM8 were electroporated into *M. tuberculosis*. Promoter activity was measured before and after long or short

Upstream region	Activity (MU) -	Activity (MU) +
$P_{Rv0277c}$	5 ± 1	5 ± 1
$P_{Rv1015c}$	6 ± 1	7 ± 1
$P_{Rv2487c}$	7 ± 1	8 ± 2

Table 3.5. Promoter activity of $P_{Rv0277c}$, $P_{Rv1015c}$ and $P_{Rv2478c}$ in *M. tuberculosis*.

ß-galactosidase activity of upstream regions of potentially antibiotic-responsive genes linked to promoterless *lacZ* during exponential growth (-) and after exposure (+) to 50 ng mL^{-1} tetracycline for 24 h is shown. The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (MU) - measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein. The background activity from pSM128 (empty vector) was 6 ± 3 MU.

Upstream region	Reporter	Stimulus	Fluorescence (RFU) -	Fluorescence (RFU) +
P_{Rv0608}	GFP-ASV	Tetracycline	6	6
	GFP-LAA	Tetracycline	8	6
P_{Rv0748}	GFP-ASV	Tetracycline	7	6
	GFP-LAA	Tetracycline	7	5
$P_{Rv3898c}$	GFP-ASV	Tetracycline	4	3
	GFP-LAA	Tetracycline	8	8
P_{whiB7}	GFP-ASV	Streptomycin	5	3
	GFP-LAA	Streptomycin	6	7

Table 3.6. Promoter activity of P_{Rv0608}, P_{Rv0748}, $P_{Rv3898c}$ and P_{whiB7} in *M. tuberculosis*.

Fluorescence of upstream regions of potentially antibiotic-responsive genes linked to two unstable versions of promoterless *gfp* during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to tetracycline (50 ng mL^{-1} for 24 h) or streptomycin (50 ng mL^{-1} for 24 h). The fluorescence of one transformant assayed in duplicate is given in Relative Fluorescence Units (RFU) - measured as fluorescence intensity per mg of protein in cell-free extracts. The background fluorescence from empty vectors pFLAME3 (carrying GFP-ASV) and pFLAME4 (GFP-LAA) was 6 RFU and 9 RFU respectively.

treatment with high or low concentrations of tetracycline or streptomycin. Due to the fact that pDS165 harbours a streptomycin resistance cassette, liquid cultures of transformants harbouring this plasmid were not subjected to treatment with streptomycin.

Weak promoter activity (~50 MU) was detected from $P_{Antigen\ 85A}$ in the absence of a stimulus. This was 5-fold higher than that of the empty vector control ($p < 0.05$) (Table 3.7). There was no significant decrease in promoter activity after treatment with tetracycline.

Fluorescence detected from $P_{myc1tetO}$ linked to both version of GFP in untreated cultures were approximately 10-fold higher than that of the background (Table 3.8). Thus the promoter was active during exponential phase and the type of tag on the GFP did not affect this. Promoter activity was not reduced in cultures subjected to treatment with tetracycline or streptomycin, regardless of the concentration used or treatment length. Hence the treatments did not interfere with synthesis of reporter protein. This data shows that none of the antibiotics interfered with translation at the concentrations used and this was irrespective of treatment length. Therefore, lack of promoter activity in the upstream regions tested was not due to the treatments interfering with protein synthesis.

Promoter activity of re-cloned upstream regions in *M. tuberculosis*

The concentrations of antibiotic and length of treatment used in this study did not interfere with translation, therefore the lack of inducibility may have been due to absence of a functional promoter in the upstream region, or a problem with the design of the plasmids. For example, the actual promoter or part of the regulatory region may not have been included in the construct. Another possibility is that the translational start site in the cloned upstream region was out of frame with the start codon of the reporter gene, resulting in the wrong ORF being translated. Therefore no translational fusion with the reporter protein would occur and promoter activity would not be picked up on in the activity assays.

To determine if vector design was responsible for the lack of induction of promoter activity, it was decided to re-clone the upstream regions of Rv1015c, Rv2487c and *whiB*6. In order to address the issue of a missing

Promoter	Stimulus	Activity (MU) -	Activity (MU) +
$P_{Antigen\ 85A}$	Tetracycline[1]	54.8 ± 6.1	46.7 ± 11.3
$P_{Antigen\ 85A}$	Tetracycline[2]	54.8 ± 6.1	49.1 ± 6.4
$P_{Antigen\ 85A}$	Tetracycline[3]	54.8 ± 6.1	48.0 ± 9.4

Table 3.7. Promoter activity of $P_{Antigen\ 85A}$ in *M. tubcerculosis* in response to different antibiotic treatments.

ß-galactosidase activity of a constitutively active promoter linked to *lacZ* during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to the following treatments: tetracycline[1] (10 µg mL^{-1} for 6 h), tetracycline[2] (50 ng mL^{-1} for 6 h) or tetracycline[3] (50 ng mL^{-1} for 24 h). The average ± standard deviation of three independent transformants assayed in duplicate is given in Miller Units (MU) - measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein. The background activity from pSM128 (empty vector) was 7 ± 3 MU.

Upstream region	Reporter	Stimulus	Fluorescence (RFU) -	Fluorescence (RFU) +
$P_{myc1tetO}$	GFP-ASV	Tetracycline[1]	59	56
	GFP-LAA	Tetracycline[1]	50	46
$P_{myc1tetO}$	GFP-ASV	Tetracycline[2]	59	53
	GFP-LAA	Tetracycline[2]	50	56
$P_{myc1tetO}$	GFP-ASV	Tetracycline[3]	59	52
	GFP-LAA	Tetracycline[3]	50	52
$P_{myc1tetO}$	GFP-ASV	Streptomycin[1]	59	52
	GFP-LAA	Streptomycin[1]	50	39
$P_{myc1tetO}$	GFP-ASV	Streptomycin[2]	59	50
	GFP-LAA	Streptomycin[2]	50	46
$P_{myc1tetO}$	GFP-ASV	Streptomycin[3]	59	49
	GFP-LAA	Streptomycin[3]	50	55

Table 3.8. Promoter activity of $P_{myc1tetO}$ in *M. tuberculosis* in response to different antibiotic treatments.

Fluorescence of a constitutively active promoter linked to two unstable versions of GFP during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to the following treatments: tetracycline[1] (10 µg mL^{-1} for 6 h) or streptomycin[1] (7.5 µg mL^{-1} for 24 h), tetracycline[2] (50 ng mL^{-1} for 6 h) or streptomycin[2] (50 ng mL^{-1} for 3.5 h) or tetracycline[3] (50 ng mL^{-1} for 24 h) or streptomycin[3] (50 ng mL^{-1} for 24 h). Fluorescence of one transformant assayed in duplicate is given in Relative Fluorescence Units (RFU) - measured as fluorescence intensity per mg of protein in cell-free extracts). The background fluorescence from empty vectors carrying GFP-ASV and GFP-LAA was 4 and 9 RFU respectively.

promoter and regulatory elements that precede it, the amino acid sequences of the proteins encoded by these three genes were subjected to a BLAST search to see if any of the homologous proteins in other organisms had a translational start site that was downstream of the annotated start site of the *M. tuberculosis* ORF. Primers for amplification of the upstream regions were designed to include less of the ORF of the preceding gene and more of the ORF of the gene itself to ensure that the most common translational start sites of the protein and its homologues were included in the region. In addition to this, primers were designed to ensure that the translational start site would be in frame with the start site of the *lacZ* gene in pSM128 (Fig 3.3). The new upstream regions were denoted $P_{nRv1015c}$, $P_{nRv2487c}$ and P_{nwhiB6} and plasmids were electroporated into *M. tuberculosis*. Promoter activity from the new plasmids was measured before and after treatment with either tetracycline, ethanol or SDS.

For upstream region $P_{nRv1015c}$, promoter activity was detected in the absence of a stimulus ($p < 0.05$) (Table 3.9). This region contains a medium strength promoter (>100 MU) which had activity picked up on once the translational start sites of the gene's ORF and the reporter gene were in frame. However, activity did not increase in response to tetracycline treatment. Very weak promoter activity (<50 MU) was detected for upstream region $P_{nRv2478c}$ in the absence a stimulus ($p < 0.05$), but this did not increase after tetracycline exposure (Table 3.9). For upstream region P_{nwhiB6}, weak to medium strength promoter activity was found in the absence of a stimulus ($p < 0.05$) (Table 3.9).

New design of the primers used to amplify the upstream regions of three genes resulted in promoter activity being detected from $P_{nRv1015c}$, $P_{nRv2487c}$ and P_{nwhiB6}. Ensuring the translational start site of the ORF of these genes is in frame with the reporter gene (which was the major difference between the previous and new primers) is critical for detection of promoter activity. However, neither of these promoters was found to be inducible under the conditions tested. These results mean that lack of activity in the other upstream regions tested may have been due to issues with vector design or there not being a functional or inducible promoter present in these regions.

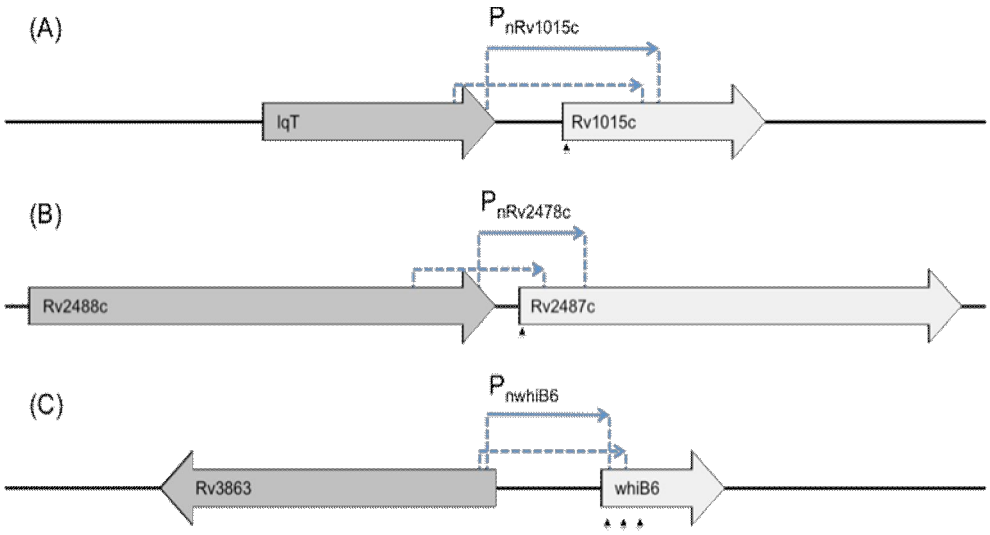

Figure 3.3 Location of primers used to amplify new upstream regions of Rv1015c, Rv2487c and *whiB6*.

Upstream regions $P_{nRv1015c}$, $P_{nRv2487c}$ and P_{nwhiB6} were amplified using new primers (solid arrow) that ensured the potential translational start sites of the ORF (black triangles) would be in frame with the translational start site of the reporter gene in the promoter probe vector. The position of the upstream region tested previously is also indicated (dashed arrow).

Upstream region	Stimulus	Activity (MU) -	Activity (MU) +
$P_{nRv1015c}$	Tetracycline	259 ± 33	279 ± 52
$P_{nRv2487c}$	Tetracycline	11 ± 1	12 ± 1
P_{nwhiB6}	Ethanol	148 ± 40	173 ± 39
P_{nwhiB6}	SDS	148 ± 40	161 ± 44

Table 3.9. Promoter activity of $P_{nRv1015c}$, $P_{nRv2487c}$ and P_{nwhiB6} in *M. tuberculosis*.

Activity of new upstream regions of Rv1015c, Rv2487c and *whiB6* linked to *lacZ* during exponential growth (-) and after treatment (+) is shown. Bacteria were exposed to the following treatments: tetracycline (10 µg mL^{-1} for 6 h), ethanol (2.5% for 1.5 h) or SDS (0.05% for 1.5 h). The average ± standard deviation of three independent transformants assayed in duplicate is given in Miller Units (MU) - measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein. The background activity from pSM128 (empty vector) ranged from 7 ± 3 MU.

3.3 Discussion

The antibiotic-inducible promoter systems currently used in mycobacteria are based on promoters or regulators from other bacteria. However, they suffer from problems such as lack of tight regulation (Blokpoel *et al.*, 2005; Carroll *et al.*, 2005; Ehrt *et al.*, 2005; Ehrt and Schnappinger, 2006). Finding and using an endogenous antibiotic-inducible promoter in *M. tuberculosis* for use in an expression system might improve control of expression. The *M. tuberculosis* genes whose upstream regions were tested for inducibility in response to antibiotics were Rv0277c, Rv0608, Rv0748, Rv1015c, Rv2487c, Rv3898c, *whiB6* and *whiB7*.

Promoter activity was only detected from P_{whiB6} in the absence of a stimulus; and from P_{Rv2487} after treatment with tetracycline. P_{Rv2487} activity was seen to be induced 2-fold after tetracycline treatment, but only to an activity slightly higher than the background. None of the other upstream regions tested were found to have any promoter activity under inducing or non-inducing conditions, even in response to the treatments originally used (Boshoff *et al.*, 2004; Geiman *et al.*, 2006). Neither using less antibiotic and keeping the treatment lengths as originally used, nor using less antibiotic but increasing treatment length, had an effect. The lack of activity was not due to the inducers inhibiting translation of the reporter genes because constitutively active plasmid controls were unaffected by the treatments used, regardless of what type of reporter gene was used.

A possible explanation for lack of promoter activity may have been that some or all of the upstream regions amplified did not contain the promoter of the gene. The promoter may have been missed due to the gene being in an operon with upstream genes or perhaps incorrect annotation of the translational start site of the open reading frames, and thus the promoter may not have been included in the regions amplified and probed for promoter activity. Alternatively, other regulatory elements further upstream of the start side may have been missed out. Another explanation for lack of activity may have been the start site of the ORF of the responsive gene being out of frame with the start site of the reporter gene, or there being too much of the ORF which would lead to production of a junk or a misfolded protein. Re-designing the primers of some of the upstream regions did

indeed result in the discovery of promoter activity for $P_{nRv1015c}$ and P_{nwhiB6}. However, none of these upstream regions were found to be inducible.

Another issue is the fact that some of the upstream regions tested in this scenario were on multicopy plasmids (pFLAME3 and pFLAME4 vector-based). Multiple copies of one promoter present within a cell might lead to dilution of the regulator of the promoter. Thus, if normally the promoter is activated through a small amount of regulator, there may not have been enough regulator available to activate all of the promoters present within the bacterium. As a consequence the promoter(s) linked to the reporter gene would not have been induced after antibiotic exposure.

An explanation for the absence of inducibility may have been the fact that both studies from which the genes were shown to be induced by streptomycin, tetracycline, SDS or ethanol show them to be so at the level of RNA only (Boshoff *et al.*, 2004; Geiman *et al.*, 2006). One of the limitations of studies using mRNA is mRNA stability. Thus, even though a gene may appear to be upregulated in terms of the level of mRNA detected in a microarray or RT-PCR study, this may be because the relevant mRNA is more stable in relation to transcripts of other genes. Hence, mRNA amount may not necessarily reflect promoter induction. In addition to this, microarray studies are often variable and data sets between different laboratories can differ depending on the methods type of probe used (Butcher, 2004).

To conclude, upstream regions of five tetracycline, one streptomycin and one ethanol and SDS-responsive gene were tested for promoter activity and inducibility. Only two of the upstream regions were found to have weak/ medium strength promoter activity and they were not found to be inducible. This may have been due to the apparent induction of the genes being a general as opposed to a specific effect, due to a multicopy plasmid effect or the upstream regions tested not containing a functional promoter.

4. Hypoxia-inducible promoters

4.1 Introduction

The emergence of drug-resistant strains of *M. tuberculosis* is an ever-increasing problem (WHO, 2009). New drugs that would shorten antibiotic treatments and target mycobacterial genes important for their survival during latent disease are urgently needed. In order to validate such genes as good drug targets one needs to control the expression of the gene under conditions believed to be encountered by the bacteria in the host such as hypoxia, nitric oxide, low pH and nutrient deprivation (Stewart, 2005; Young, 2005). Finding a mycobacterial promoter that responds to these stimuli would enable construction of an inducible promoter system that can be employed to regulate expression of a gene of interest under these conditions.

4.1.1 The Wayne model of non-replicating persistence

The precise nature of the conditions encountered by bacilli during the latent stage of disease is unknown, but several studies have established a link between mycobacterial persistence during latency and hypoxia (Aly *et al.*, 2006; Flynn and Chan, 2001; Kaplan *et al.*, 2003; Rustad *et al.*, 2009; Via *et al.*, 2008). The mechanisms by which mycobacteria persist during, and reactivate from, a latent infection are poorly understood. Several *in vivo*, *ex vivo* and *in vitro* models have been developed to mimic the conditions encountered by the bacteria during this stage of disease during which they are believed to adopt a non-replicating state (Young, 2005). Amongst the *in vitro* models of hypoxia the Wayne model of hypoxia is the most frequently used (Rustad *et al.*, 2008; Sherman *et al.*, 2001; Wayne, 1977; Wayne and Lin, 1982; Wayne and Hayes, 1996). Wayne and his coworkers developed an *in vitro* model of dormancy based on the premise that the bacteria experience oxygen starvation during latent infection (Wayne and Hayes, 1996). They showed that rapidly decreasing the amount of oxygen available to the cultures resulted in cell death, whereas allowing the bacteria to slowly adapt to reduced oxygen tensions enabled them to enter a non-replicating state (Wayne and Lin, 1982). They observed two distinct stages: Non-Replicating Persistence stage 1 (NRP1), which occurred under microaerophilic conditions (oxygen concentration around 1%); and Non-replicating Persistence stage 2 (NRP2), which occurred at the onset of

anaerobic conditions (oxygen concentration below 0.06%) (Wayne and Hayes, 1996). NRP1 is characterised by cell enlargement due to thickening of the cell wall, a significant decrease in RNA synthesis, and termination of cell division and DNA synthesis (Cunningham and Spreadbury 1998; Muttucumaru *et al.,* 2004; Wayne and Hayes 1996). In NRP2, cell enlargement ceases and the antibiotic susceptibility profile of the cells changes (Wayne and Hayes, 1996). Gene expression profiles of these stages as well as during a steady state model of hypoxia showed that there is a distinct temporal expression of genes as the cells adapt (Bacon *et al.,* 2004; Muttucumaru *et al.,* 2004; Voskuil *et al.,* 2004).

In vitro models of hypoxia have shown that adaptation to hypoxia is accompanied by distinct physiological, structural, metabolic and molecular changes within the bacteria. These changes include enhanced utilisation of the glyoxylate shunt pathway, together with an increase in the reductive amination of glyoxylate (Wayne and Lin, 1982), enhanced resistance to conventional antitubercular drugs, susceptibility to metronidazole (Wayne and Hayes, 1996), loss of acid fastness (Gillespie *et al.,* 1986; Wayne and Sramek 1994), induction of HspX (Yuan *et al.,* 1996), and thickening of the cell wall (Cunningham and Spreadbury, 1998).

4.1.2 Mycobacterial response to hypoxic conditions

Growth of mycobacteria under hypoxic conditions has been studied extensively and adaptation to hypoxia can be divided into an early, transient response (during which the DosR regulon is upregulated), followed by the enduring hypoxic response (Rustad *et al.*, 2009).

The DosR regulon

The DosR or dormancy regulon comprises a set of about 50 genes that are highly induced by hypoxia, nitric oxide (NO) and during macrophage infection, and are regulated by the Dos two-component regulatory system (Bacon *et al.,* 2004; Dasgupta *et al.,* 2000; Florczyk *et al.,* 2003; Kumar *et al.,* 2007; Ohno *et al.,* 2003; Park *et al.,* 2003; Sherman *et al.,* 2001; Sousa *et al.,* 2007; Voskuil *et al.,* 2003; Voskuil *et al.,* 2004).

There are many highly specific regulators of gene expression, for example the Lac repressor of *E. coli*, which provide a simple means of gene regulation (Watson, 2004). There are also more complex forms of

regulation involving two-component regulatory systems (2CRs) and the use of alternative sigma factors to alter the expression of different sets of genes (Rison 2005; Watson, 2004). 2CRs consist of a sensor and a response regulator (Fig 4.1). The sensor kinase, usually membrane bound, consists of an N-terminal sensor domain and a C-terminal transmitter domain. Detection of an environmental stimulus via the sensory domain leads to autophosphorylation of a conserved histidine residue in the transmitter domain of the sensor kinase. Phospho-relay allows activation of the response regulator. The response regulator is a DNA binding protein that acts as a transcription factor and can turn sets of genes on or off. Genes that are directly controlled by the same regulator in this fashion are called regulons. In addition to direct control, regulatory cascades can occur where a regulator controls the expression of another, thus magnifying the number of genes controlled in response to the original signal (Barrett, 1998; Rison, 2005). So far 12 2CRs have been identified in *M. tuberculosis*, some of which play a key role in virulence, and there are also orphan sensors and regulators where the corresponding component of the 2CR has not been assigned (Morth *et al.,* 2005; Rison 2005; Ryndak *et al*., 2008).

DosR/S/T - encoded by Rv3133c, Rv3132c and Rv2027c respectively - is an unusual 2CR system. The response regulator DosR can be phosphorylated by two sensor kinases: DosS and DosT. Interestingly, *dosS* is part of the dormancy regulon, whereas *dosT* is not (Sherman *et al.*, 2001; Voskuil *et al.*, 2003). DosS and DosT are oxygen-switched kinases, both are heme binding and contain GAF (cGMP, adenylyl cyclase, FhlA) domains (Sardiwal *et al.,* 2005; Sousa *et al.,* 2007). Recently, the mechanism of signal sensing of this system has been unveiled (Fig 4.2).

DosS is a redox sensor and DosT is a hypoxia sensor (Kumar *et al.*, 2007). Under normoxic conditions both DosS and DosT become saturated with O_2 and their kinase activity is switched off. Under hypoxic conditions, the kinases exist predominantly in the reduced (DosS) and ferrous (DosT) state and are active; they autophosphorylate and transfer the phosphate onto DosR. Thus, the activated DosR will induce the DosR regulon (Roberts *et al.*, 2004). Induction of the regulon by NO and carbon monoxide (CO) can be explained by two models. One model proposes that binding of these divalent gases to the heme irons of the sensors displaces O_2 (Kumar *et al.,* 2007; Sousa *et al.,* 2007). Another model proposes that the gases can only

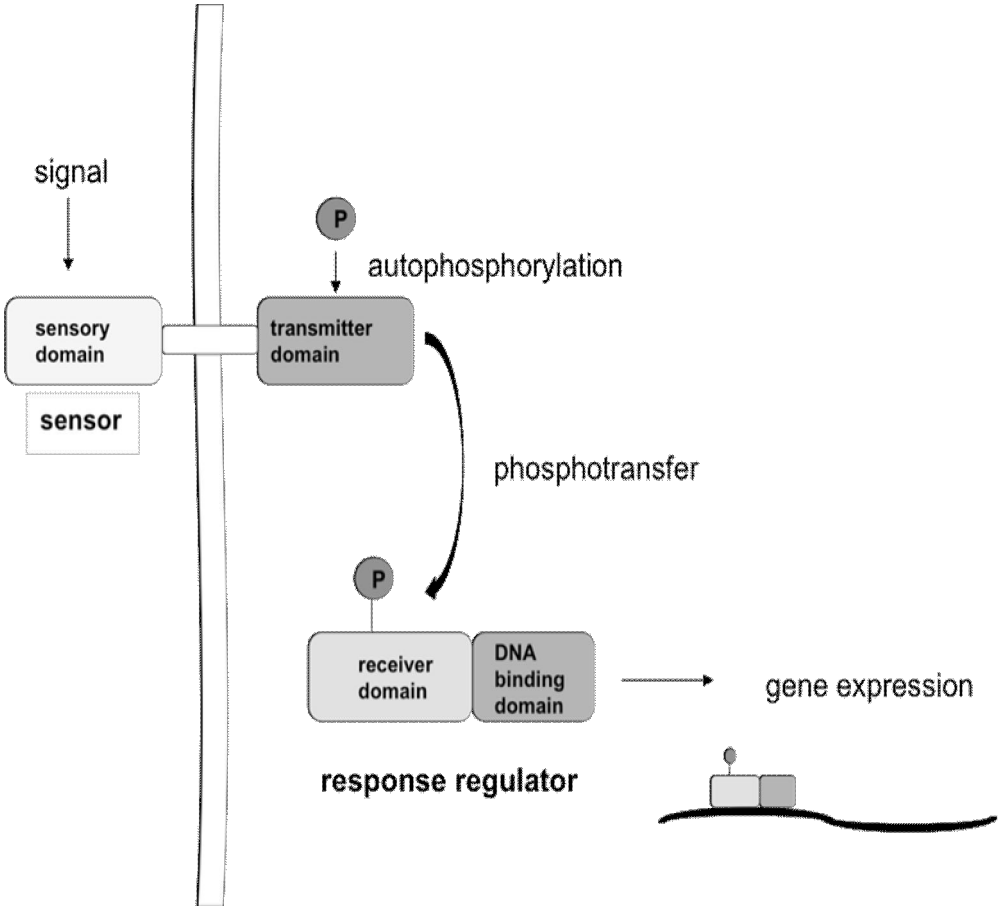

Figure 4.1. The 2CR paradigm.

The signal causes the membrane-bound sensor to autophosphorylate. The phosphate is transferred to the regulatory protein via a phosphotransferase reaction. Phosphorylation of the regulator activates the DNA binding domain and enables the regulator to act as a transcription factor to change gene expression. Adapted from Rison, 2005.

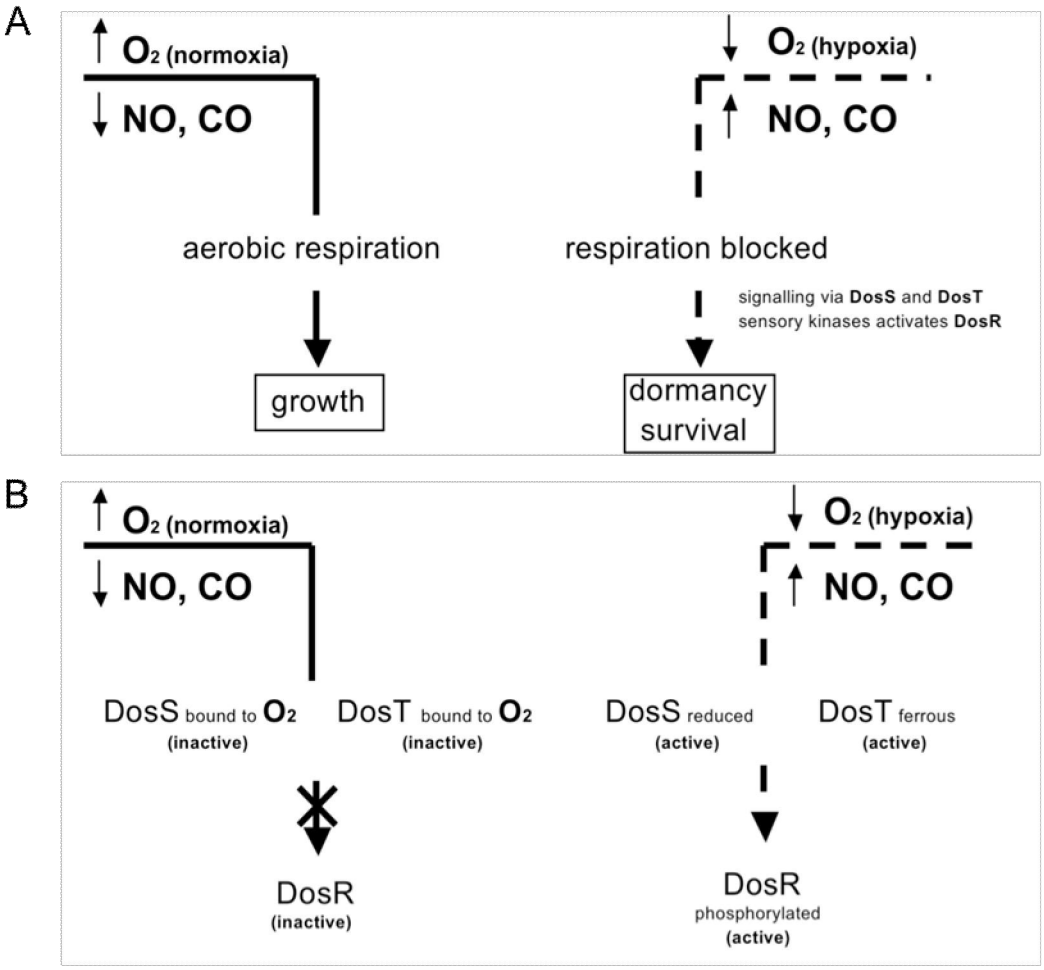

Figure 4.2. Schematic depiction of the regulation of the dormancy response in *M. tuberculosis*.

(A) Control of respiration by hypoxia, NO and CO. (B) Control of the DosR/S/T regulatory system by hypoxia (through the redox sensor DosS and the hypoxia sensor DosT) and by NO and CO (through DosT). Once activated, the response regulator DosR upregulates expression of the dormancy regulon. Adapted from Voskuil, 2009.

bind ferrous iron in DosT (Kumar *et al.*, 2007). In either case it is postulated that displacing O_2 by the high affinity binding of NO or CO to the heme locks the kinase in an active state.

DosR is the transcriptional regulator required for induction of the DosR regulon (Sherman *et al.*, 2001). DosR is a classic two-component system regulator with an N-terminal receiver domain involved in phospho-relay and a C-terminal DNA binding domain that contains a helix-turn-helix motif (Dasgupta *et al.*, 2000). The site of phosphorylation by its sensor kinases has been mapped to Asp54 (Park *et al.*, 2003; Roberts *et al.*, 2004). The residues critical for phosphorylation of DosR, as well as DNA binding activity have also been mapped and a DNA binding motif to which DosR binds (DosR motif) has also been identified (Florczyk *et al.*, 2003; Park *et al.*, 2003; Wisedchaisri *et al.*, 2005). DosR motifs have been identified upstream of several genes in the dormancy regulon. The sequence of the motif is a 20 bp palindrome with the consensus sequence 5'-TTSGGACTWWAGTCCCSAA-3' (S= C/G and W= A/T), a variant of which is found upstream of almost half of the *M. tuberculosis* genes that are rapidly induced by hypoxia (Florczyk *et al.,* 2003; Park *et al.,* 2003).

Data on the importance of DosR during infection is conflicting. Deletion strains of DosR have been constructed, but results of *in vivo* experiments investigating the importance of DosR during host infection differed considerably. One study has shown a DosR mutant to be hypervirulent (Parish *et al*., 2003), whereas another study found a DosR mutant strain to be attenuated in virulence (Malhotra *et al.*, 2004). Two recent studies have shown DosR to be non-essential for virulence during several *in vivo* infection models, but a third study found DosR and DosS to be required for virulence in animal models (Bartek *et al.*, 2009; Converse *et al.*, 2009; Rustad *et al.*, 2008). In light of this, it is interesting to note that analysis of the hypervirulent W-Beijing strain of *M. tuberculosis* showed that some members of the DosR regulon were constitutively overexpressed, indicating that the regulon plays a significant role during infection (Reed *et al.*, 2007).

The extended hypoxic response (EHR)

Until recently, the DosR regulon was thought to be key for entry into and survival of a non-replicating state. However, a study carried out by Rustad and colleagues in 2008 using a DosR knockout strain showed that DosR

was dispensable for bacterial virulence in three strains of mice (Rustad *et al.*, 2008). The DosR regulon was orginally defined as the set of genes upregulated after two hours in a defined hypoxia model (Park *et al.*, 2003; Sherman *et al.*, 2001). A microarray study carried out by Rustad and colleagues, monitoring gene expression of *M.*

tuberculosis exposed to hypoxia for longer time periods, found that expression of most of the genes of the DosR regulon was only transitory, and that half of the regulon was no longer significantly induced after 24 hours of hypoxia (Rustad *et al.*, 2008). In addition to this, a second transcriptional response consisting of 230 genes - not initially induced, but upregulated after 4-7 days of hypoxia - was observed. The authors termed this pattern of gene expression the 'enduring hypoxic response' (EHR). Whilst the function of the majority of these genes remains unknown, a large proportion are putative regulatory proteins. Unlike the DosR regulon, the genes that form part of the EHR overlap with genes that are upregulated under conditions of nutrient starvation (Rustad *et al.*, 2008). This suggests that the EHR contributes to the adaptation to and maintenance of a non-replicating state (Rustad *et al.*, 2008; Rustad *et al.*, 2009).

4.1.3 Model organisms: *M. smegmatis*

Despite the recent advancements in genetic tools available for studying *M. tuberculosis*, it remains a difficult organism to work with. Related mycobacteria that do not cause disease in humans and grow faster have proven more tractable and are increasingly being used as model organisms to help understand the complex biology that underlies how *M. tuberculosis* causes disease.

M. smegmatis was first isolated in 1824 by Lustgarten and in 1885 isolated from human smegma by Alvarez and Tavel (Alvarez, 1885; Lustgarten, 1884). It is a fast-growing non-pathogenic mycobacterium and has a generation time of 3-4 hours compared to 24 hours for *M. tuberculosis*. It does not infect humans and it does not require ACDP Hazard Group 3 containment. It is frequently used as a model organism for other mycobacterial species (Reyrat, 2001). Whilst it is useful for general research of mycobacterial qualities and can be used to distinguish species-specific genes and their function, one has to bear in mind that it is

saphrophytic and not pathogenic, and cannot used as a model for pathogenesis (Reyrat, 2001).

The genome of *M. smegmatis* has been partly sequenced and is about 1.7 times bigger than that of *M. tuberculosis* (Reyrat, 2001). *In silico* comparisons between the unfinished *M. smegmatis* genome sequence (www.tigr.org) and that of *M. tuberculosis* allowed the identification of many conserved two-component regulatory systems and sigma factors.

The physiological behaviour of *M. smegmatis* during the Wayne model mirrors the characteristics of *M. tuberculosis*. Slow depletion of oxygen through a self-generated oxygen gradient permits adaptation to hypoxia resulting in a non-replicating state, whereas abrupt depletion leads to rapid cell death (Dick *et al.*, 1998). The anaerobic *M. smegmatis* bacilli exhibit an antibiotic susceptibility pattern similar to that of non-replicating tubercule bacilli, becoming sensitive to metronidazole and resistant to the gyrase inhibitor ofloxacin. Another key feature of *M. tuberculosis* dormancy, the synchronisation of the culture due to arrest at a uniform stage in the cell cycle and termination of DNA synthesis, as seen during the Wayne model, has also been observed in anaerobic *M. smegmatis* (Dick *et al.*, 1998).

Homologues of DosR-DosS (DevR and DevS) and several other genes that are upregulated under microaerophilic conditions in *M. tuberculosis* have been identified in *M. smegmatis* (Tyagi and Sharma, 2002). Experiments using RT-PCR and immunogold electron microscopy have shown the operon consisting of Ms3134c, *devR and devS* is induced approximately 5-fold and continuously expressed during hypoxic conditions (Mayuri, *et al.,* 2002). Analysis of protein expression in a *devR* mutant strain revealed the presence of five proteins whose expression under hypoxic conditions is dependent on *devR*. One of these proteins was found to be a homologue of HspX (O'Toole *et al.,* 2003).

The *M. smegmatis* DevR protein is 84% identical at the amino acid level to *M. tuberculosis* DosR. The site of phosphorylation of DosR (Asp54), as well as the residues implicated in DosR phosphorylation activity (Asp8, Asp9 and Lys104) - and the DNA binding regions (Lys179, Lys182 and Asn183) - are conserved between the two (Wisedchaisri *et al.,* 2005) (Fig. 4.3). The homologue of the sensor kinase DosS, DevS, is 66% identical at the amino acid level and the site of DosS autophosphorylation (His395) is conserved

Figure 4.3. Amino acid alignment of *M. tuberculosis* DosR (DosR$_{Mtb}$) and *M. smegmatis* DevR (DevR$_{Msm}$).

Identical amino acids are marked by an asterisk. The site of phosphorylation of DosR (Asp54) and the residues involved in DNA binding (Lys179, Lys182 and Asn183) are shaded.

(Wisedchaisri *et al.,* 2005). However, DevS has none of the three transmembrane domains predicted to be present in DosS (Mayuri *et al.,* 2002). Taking these findings together, *M. smegmatis* appears to be a very adequate model of *M. tuberculosis* with respect to the hypoxic response.

4.1.4 Potential promoters investigated in this study

One of the aims of this study was to construct an inducible promoter system that could be employed to regulate expression of a gene of interest during an *in vivo* model. In order to find a mycobacterial promoter that is upregulated by the stimuli encountered during host infection, for example hypoxia (Aly *et al*., 2006), genes from the DosR regulon were picked as potential candidates and their upstream regions tested for promoter activity.

Data from the literature was mined to find members of the DosR regulon that were the most highly induced in response to hypoxia, NO treatment or under standing culture conditions (Bacon *et al*., 2004; Kendall *et al*., 2004b; Ohno *et al*., 2003; Park *et al*., 2003; Sherman *et al*., 2001; Voskuil *et al*., 2003; Voskuil *et al*., 2004; Yuan *et al*., 1998). The genes selected for this study were Rv2627c, Rv2626c, Rv2625c and *hspX* (also known as Rv2031 or *acr*). Microarray studies have shown these genes to be consistently upregulated under various conditions such as treatment with a NO donor, hypoxia and during stationary phase (Table 4.1) (Bacon *et al.,* 2004; Kendall *et al.,* 2004; Ohno *et al.,* 2003; Sherman *et al.,* 2001; Voskuil *et al.,* 2003; Voskuil *et al.,* 2004; Wayne and Hayes, 1996; Wayne and Hayes, 1998).

The genomic localisation of Rv2627c, Rv2626c and Rv2625c suggests that these genes may form an operon (Fig 4.4 on page 103). Rv2627c encodes a conserved hypothetical protein of unknown function. Rv2626c (also known as Hypoxic Response protein 1 or HRP1) encodes an unusual 'CBS domain only' protein that is sensitive to proteolytic cleavage at its C-terminus and is able to dimerise with itself. HRP1 is secreted by *M. tuberculosis* under standing culture conditions, despite an apparent lack of secretion signals (Sharpe *et al*., 2008). However, the biological function of Rv2626c is unknown. Rv2625c, the smallest of the genes of this putative operon, is predicted to encode a zinc metallopeptidase with a transmembrane protein and a cystathionine-ß-synthase (CBS) domain

Condition	Rv2627c	Rv2626c	Rv2625c	*hspX*	Source
Hypoxia[1]	17	37.4	6.3	13.6	(Sherman *et al*., 2001)
	12.4	24.5	3	27.9	(Park *et al*., 2003)
	11.9	40.6	6.9	14.6	(Voskuil *et al*., 2004)
Hypoxia[2]	15.1	56.5	5.3	31	(Voskuil *et al*., 2004)
Hypoxia[3]	7.2	12	8.5	24.2	(Bacon *et al*., 2004)
Wayne model[4]	47	107	17	71	(Voskuil *et al*., 2004)
Wayne model[5]	29	63	11	47	(Voskuil *et al*., 2004)
Aerobic growth[6]	7	23	2	28	(Voskuil *et al*., 2004)
Aerobic growth[7]	3.4	33	2.3	13	(Voskuil *et al*., 2004)
NO[8]	15.3	20.9	8.8	24.7	(Voskuil *et al*., 2003)
NO[9]	191.4	n.g.	42.6	171.5	(Ohno *et al*., 2003)
Standing culture*	2.4	4.3	2.7	83.2	(Kendall *et al*., 2004)

Table 4.1 Fold-induction of Rv2627c, Rv2626c, Rv2625c and *hspX* from microarray studies profiling DosR regulon expression in *M. tuberculosis*.

Details of the conditions and *M. tuberculosis* strains are outlined below.

Hypoxia:[1] steady state model of hypoxia, 0.2% O_2 for 2 h (H37Rv); [2] steady state model of hypoxia, 0.2% O_2 for 4 d (H37Rv); [3] chemostat culture, 1% O_2 (H37Rv). **Wayne model:** [4] non-replicating phase for 6 d (1254); [5] non-replicating phase for 14 d (1254). **Aerobic growth**: [6] 14 d (1254); [7] 24 d (1254). **NO**: [8] 50 µM DETA/NO for 40 min (H37Rv); [9] 100 µM SPER/NO for 4 h (*Erdman*). **Standing culture**: * for 30 min (H37Rv).

(Sharpe *et al.*, 2008). Unpublished data suggests that it could be involved in gene regulation, since several genes normally upregulated during hypoxia are not induced in a Rv2625c deletion strain (Parish unpublished).

HspX encodes the 14 kDa alpha-crystallin protein, an ATP-dependent chaperone associated with the mycobacterial cell wall, and a prominent marker of the hypoxic response as well as stationary growth phase (Cunningham and Spreadbury 1998; Sherman *et al.,* 2001; Yuan *et al.,* 1998). Multiple stresses such as hypoxia and standing, as well as treatment with NO donors have been shown to induce its expression in *M. tuberculosis* (Bacon *et al.,* 2004; Kendall *et al.,* 2004b; Ohno *et al.,* 2003; Voskuil *et al.,* 2003; Voskuil *et al.,* 2004; Yuan *et al.,* 1996; Yuan *et al.,* 1998). *HspX* is also expressed during latent infection as antibodies against it have been isolated from patient sera and it is also important for replication during macrophage infection (Lee *et al.,* 1991; Verbon *et al.,* 1992; Yuan *et al.,* 1996).

A consensus for the DosR motif has been characterised, and upstream regions of Rv2627c, Rv2626c and *hspX* contain DosR motifs in their promoter regions (Park *et al.,* 2003). The upstream regions of Rv2627c, Rv2626c and *hspX* contain two DosR motifs, each of which have strong matches to the DosR motif consensus (Park *et al.,* 2003). Spacing between the DosR motifs differs between the three upstream regions (Park *et al.,* 2003) the Rv2627c upstream region - which contains one of the DosR motifs with the best match to the consensus - the two motifs are separated by 28 bp. In the Rv2626c upstream region, motifs are located three bp apart, and in the *hspX* upstream regions the motifs are separated by 37 bp. The interaction between DosR and the *hspX* promoter was characterised further. Mutating either one of the DosR motifs severely reduced promoter induction during hypoxia - a 28-fold induction being reduced to 16-fold if the distal motifs was mutated, and to 5-fold when the proximal motif was mutated. Promoter induction was lowered to a 2-fold change when both motifs were mutated. This indicates that DosR binding is necessary for promoter activity in response to hypoxia, but not during aerobic growth conditions (Park *et al.,* 2003).

The DosR regulon members Rv2627c, Rv2626c, Rv2625c and *hspX* were selected as candidate genes for further study, because robust induction of

these genes in response to a variety of stimuli (Table 4.1) might make their promoters suitable for use in an inducible expression system.

4.2 Results

4.2.1 Construction of plasmids

This study investigated if inducible promoter activity was present in the upstream regions of Rv2627c (P_{27}), Rv2626c (P_{26}), Rv2625c (P_{25}) and *hspX* (P_{hspx}) in response to ethanol or DETA/NO (an NO donor) treatments, and under standing or hypoxic culture conditions in *M. smegmatis* and *M. tuberculosis.*

For each of the genes selected, the region upstream of the predicted translational start site was cloned into the promoter probe vector pSM128 (Dussurget *et al.,* 1999). The amplified upstream regions of Rv2627c, Rv2626c and Rv2625c are referred to as P_{27}, P_{26} and P_{25} respectively and the upstream region of *hspX* is denoted as P_{hspx} (Fig 4.4).

4.2.2 Promoter activity in *M. smegmatis*

Promoter activity in response to standing, ethanol, and DETA/NO treatment

Previous work in *M. tuberculosis* suggested that DosR is expressed in response to standing culture, and after exposure to DETA/NO or ethanol (Kendall *et al.,* 2004b; Voskuil *et al.,* 2003). Since *M. smegmatis* has a DosR homologue (DevR) and displays a hypoxic response similar to that of *M. tuberculosis*, it was decided to work with *M. smegmatis* as a model initially in order to produce faster but comparable results to *M. tuberculosis* (Dick *et al.*, 1998; Mayuri *et al.*, 2002).

Promoter activity was assayed in response to treatment with ethanol, treatment with NO or standing culture conditions (Fig 4.5). Only plasmids containing the regions upstream of Rv2626c, Rv2625c and *hspX* were electroporated into and tested in *M. smegmatis*. Data on the upstream region of Rv2627c is missing because cloning was not completed at the same time as cloning of the other regions.

For region P_{26}, very weak promoter activity of 39 MU was detected ($p < 0.05$) (Fig 4.5). Promoter activity did not increase in cultures subjected to

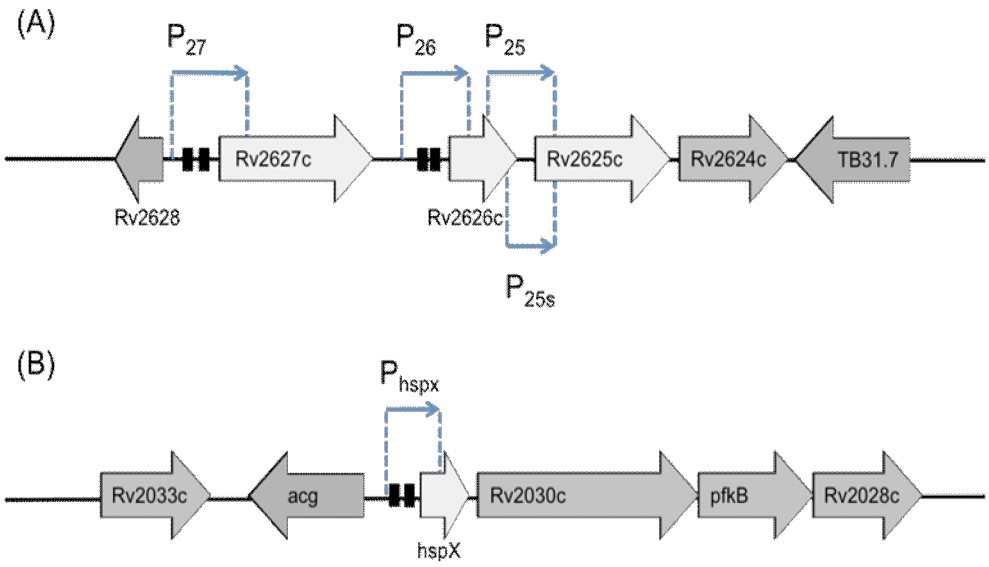

Figure 4.4. Genomic organisation of Rv2627c-Rv2625c and *hspX* in *M. tuberculosis*.

Light grey shaded arrows represent the open reading frames of Rv2625c, Rv2626c and Rv2627c (A) and *hspX* (B). DosR motifs are indicated by black boxes. The upstream regions that were amplified by PCR and cloned into the promoterless expression vector pSM128 are indicated and denoted as P_{27} (323 bp), P_{26} (438 bp), P_{25} (350 bp), P_{25s} (180 bp) and P_{hspx} (256 bp).

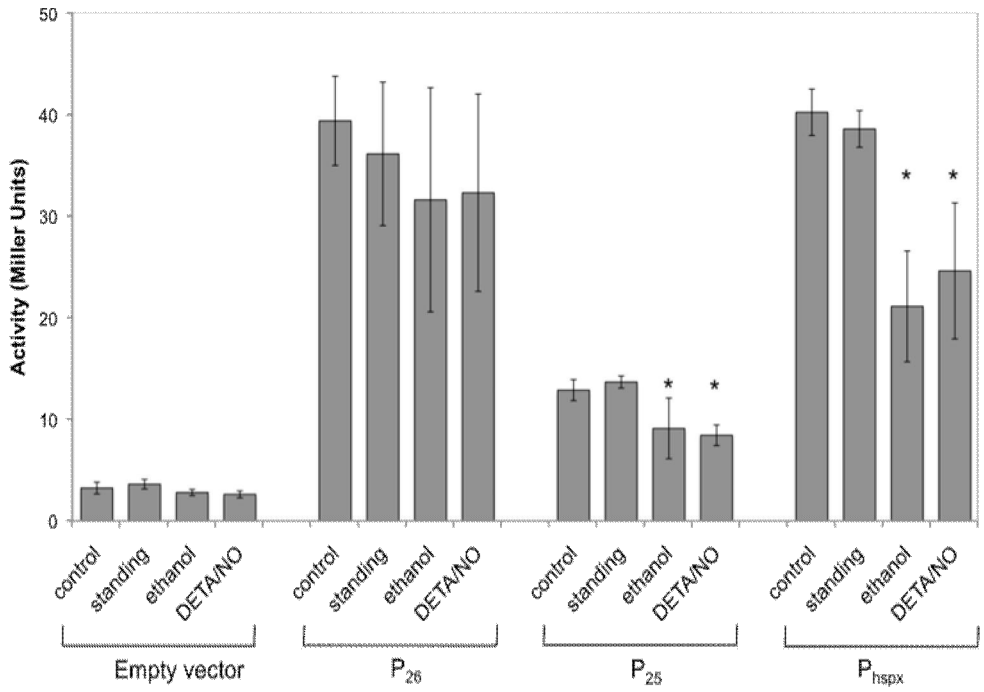

Figure 4.5. Promoter activity for P_{26}, P_{25} and P_{hspx} in response to standing culture conditions, ethanol or DETA/NO treatment in *M. smegmatis*.

ß-galactosidase activity in liquid cultures of *M. smegmatis* is shown. Activity in 24 h liquid cultures is shown before treatment (control) and after cultures were subjected to standing culture conditions or treatment with 5% ethanol or 50 µM DETA/NO for 30 min. The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein). A significant difference compared to the untreated control is marked by an *($p < 0.05$) for each promoter.

standing conditions, ethanol treatment or DETA/NO treatment. This indicates that P_{26} does contain a promoter that functions in *M. smegmatis*, but that the promoter is not induced by any of the stimuli tested. For region P_{25}, extremely weak promoter activity of 13 MU was found in the absence of a stimulus ($p < 0.05$) (Fig 4.5). Promoter activity remained the same in standing culture conditions but was significantly reduced in cultures that were treated with ethanol or DETA/NO. For P_{hspx}, promoter activity in the absence of a stimulus was 40 MU ($p < 0.05$) (Fig 4.5). Promoter activity decreased after ethanol or DETA/NO treatment. These results show that all upstream regions contain promoters that are functional, but not inducible in *M. smegmatis*.

To determine if the repression of P_{25} and P_{hspx} activity was due to a toxic effect of ethanol and DETA/NO treatment, samples of the culture both before and after addition of the stimuli were plated for colony count determination. There was no significant difference in the number of colonies obtained before and after treatment, indicating that the reduction of activity was not due to the loss of viable bacteria in the culture as a result of a bactericidal effect of the treatments.

Promoter activity during stationary phase

Stationary phase is defined as the time when the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust the resources available to them. In *M. tuberculosis*, DosR as well as Rv2626c, Rv2625c and *hspX* are upregulated after 14-24 d of aerated growth, when the bacteria reach stationary phase (Voskuil *et al.*, 2004). To compare potential differences in promoter activity between exponential growth phase (OD_{600} <1.0) and stationary growth phase (OD_{600} >1.0), liquid cultures of transformants were grown for 16 h (OD_{600} 0.7 to 1.0) and 72 h (OD_{600} ~2.0) in Lemco medium.

There was no difference in activity of P_{26} between 16 h and 72 h old cultures (Fig 4.6), thus the promoter was not induced during stationary phase growth. Activity of P_{25} was significantly lower in cultures grown for 72 h as opposed to 16 h old cultures ($p < 0.05$). Therefore, unexpectedly, P_{25} was repressed rather than induced in stationary phase *M. smegmatis*. Activity of P_{hspX} did not increase in cultures grown for 72 h, thus neither P_{26} nor P_{hspx} were induced in stationary phase in *M. smegmatis*.

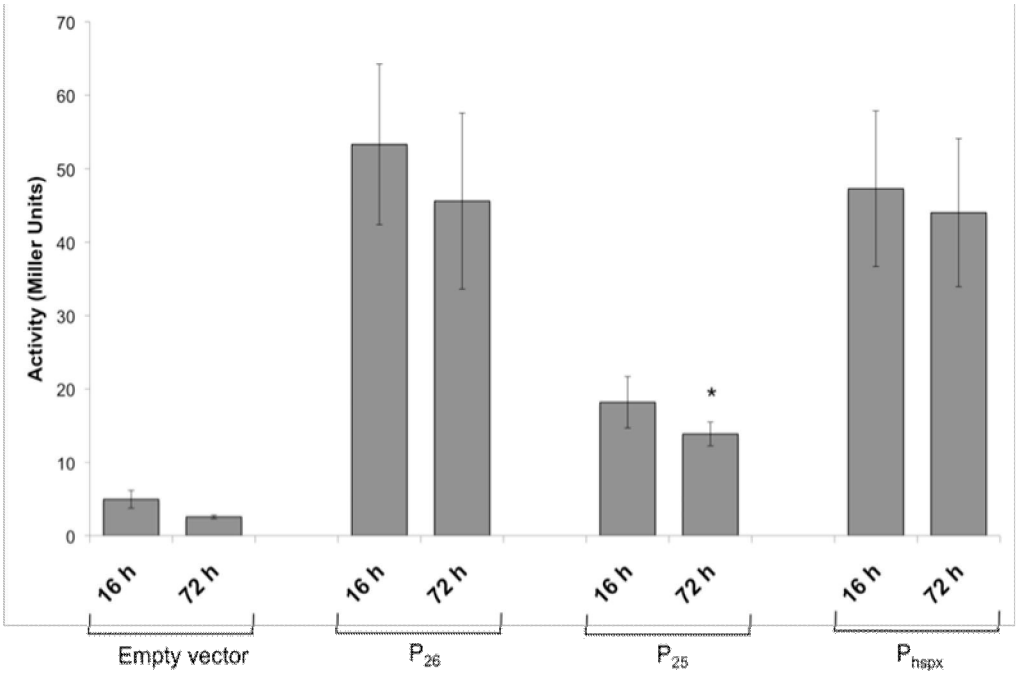

Figure 4.6. Promoter activity for P_{26}, P_{25} and P_{hspx} during exponential and stationary growth phase in *M. smegmatis*.

ß-galactosidase activity in liquid cultures of *M. smegmatis* is shown. Activity in 16 h exponential phase and 72 h stationary phase liquid cultures is shown. The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein). Background activity from pSM128 phase was 5 ± 1 MU during exponential growth and 3 ± 1 MU during stationary phase. A significant difference compared to the 16 h control is marked by an * ($p < 0.05$) for each promoter.

Promoter activity during the Wayne model of hypoxia

No induction of promoters was observed during stationary phase in *M. smegmatis*. According to various studies, Rv2626c, Rv2625c and *hspX* are induced during hypoxia in *M. tuberculosis* (Bacon *et al.*, 2004; Park *et al.*, 2003; Sherman *et al.*, 2001; Voskuil *et al.*, 2003; Voskuil *et al.*, 2004). Since adaptation to hypoxic conditions is conserved between *M. tuberculosis* and *M. smegmatis* (Dick *et al.,* 1998; Mayuri *et al.,* 2002; O'Toole *et al.,* 2003), it was decided to test if hypoxia induced promoter activity in *M. smegmatis*.

Compared to promoter activity during aerobic growth, activity of P_{26}, P_{25} and P_{hspx} was repressed during microaerophilic conditions (day 3) and anaerobic conditions (day 5) ($p < 0.05$) (Fig 4.7). P_{26} activity was almost half of what had previously been observed and P_{25} activity was reduced to levels of the empty vector control. P_{26} and P_{25} activities were the same at day 3 and day 5. P_{hspx} activity at day 3 was less than half than during aerobic growth and decreased even further at day 5. Thus, hypoxia failed to induce activity of P_{26}, P_{25} or P_{hspx} in *M. smegmatis.*

4.2.3 Promoter activity in *M. tuberculosis*

Promoter activity in response to standing, ethanol and DETA/NO treatment

Application of the treatments in *M. smegmatis* did not indicate which of the promoters studied would be most suitable for an inducible promoter system because, surprisingly, none of the promoters tested were inducible. Since the system is aimed to work in *M. tuberculosis,* it was decided to investigate the activity of the promoters in *M. tuberculosis* directly. Promoter activity in response to treatment with ethanol, DETA/NO or in standing culture conditions was measured (Fig 4.8).

P_{26} is a medium strength promoter (100-2000 MU) in *M. tuberculosis.* In the absence of stimuli promoter activity was 88 MU ($p < 0.05$) (Fig 4.8) and increased in response to standing culture conditions, ethanol and the DETA/NO (755 MU, 509 MU and 1681 MU respectively) (Fig 4.8). However, there was a high variation between transformants when the stimuli were applied, and there was no statistically significant difference in activities after DETA/NO treatment.

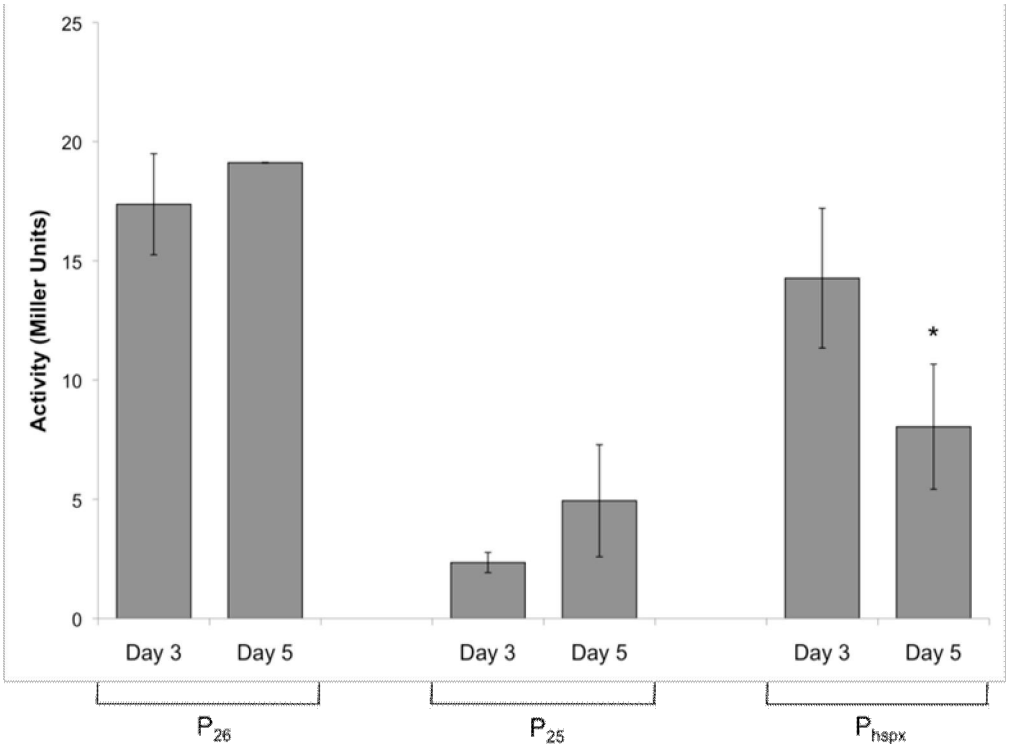

Figure 4.7. Promoter activity of P_{26}, P_{25} and P_{hspx} in response to hypoxia in *M. smegmatis*.

The experiment was conducted in Dubos-Tween-Albumin (DTA) medium. Liquid cultures were inoculated to a starting OD_{600} of 0.04. Methylene blue was used as an indicator of oxygen concentration in the medium. The dye faded after 3 d (microaerophilic conditions) and appeared colourless after 5 d (anaerobic conditions). The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein). A significant difference compared to the microaerophilic conditions is marked by an * ($p < 0.05$) for each promoter.

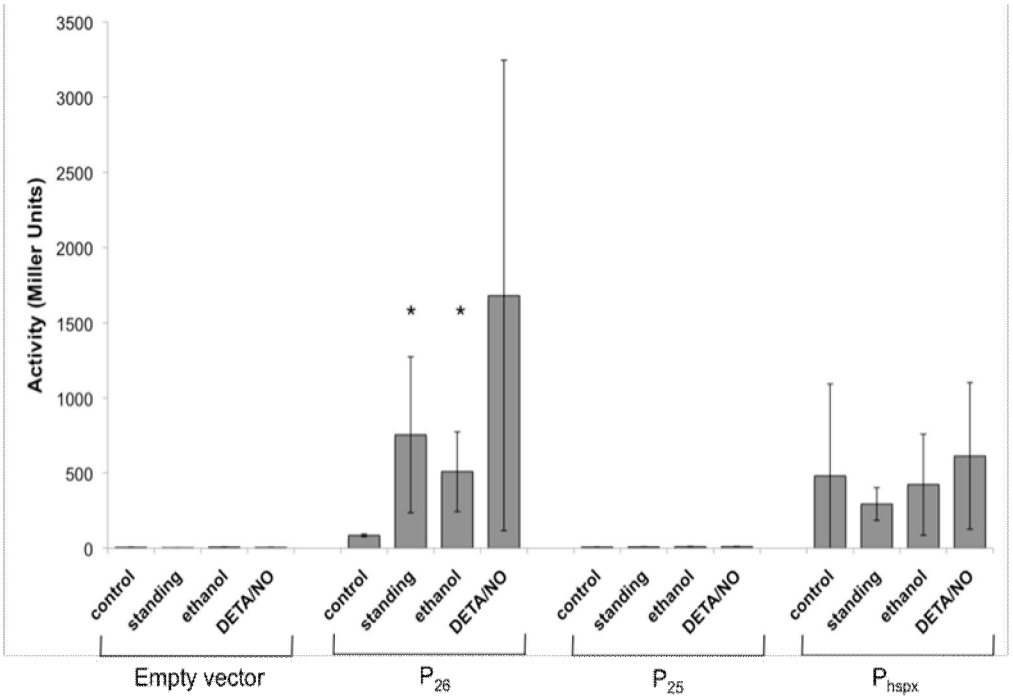

Figure 4.8. Promoter activity for P_{26}, P_{25} and P_{hspx} in response to standing culture conditions, ethanol or DETA/NO treatment in *M. tuberculosis*.

ß-galactosidase activity in liquid cultures of *M. tuberculosis* transformed with pSM128 (empty vector) and promoter probe vectors is shown. Activity in 7 d liquid cultures is shown before treatment (control) and after cultures were subjected to standing culture conditions or treatment with 5% ethanol or 50 µM DETA/NO for 60 min. The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein). A significant difference compared to the untreated control is marked by an * ($p < 0.05$) for each promoter.

Activity of P_{25} varied from 7-10 MU which was only slightly higher than the background ($p < 0.05$) (Fig 4.8). Promoter activity was not induced by standing culture conditions, or treatment with ethanol or DETA/NO. The P_{hspx} region displayed medium strength promoter activity of 480 MU in the absence of stimuli, but variation between the transformants was very high (Fig 4.8). Promoter activity under standing culture conditions, in the presence of ethanol and in the presence of DETA/NO was not significantly different from this basal activity due to the high variation between transformants in all of the conditions assayed.

The high variation in the results obtained for P_{26}, when the stimuli were applied may be attributed to variation in response to treatment between individual transformants. Some transformants were induced more in response to stimulation and thus the standard deviation between the three transformants was high. Variation in activity of P_{hspx} was also high even in the absence of a stimulus. This may be explained by the fact that DosR is expressed in stationary phase (OD_{580} of 1.0 or higher) (Voskuil *et al.*, 2004). When testing for inducible promoter activity in response to stress stimuli, cultures were not inoculated with a particular starting OD_{580} and the OD_{580} of the cultures was not measured before application of the treatments. It is possible that some liquid cultures had already reached stationary phase, and thus DosR was being expressed and the promoter already switched on. This would explain the high variation of P_{hspx} activity in the absence of a stimulus.

Promoter activity during the Wayne model in *M. tuberculosis*

Rv2626c, Rv2625c and *hspX* are upregulated by hypoxia in *M. tuberculosis* (Bacon *et al.,* 2004; Sherman *et al.,* 2001; Voskuil *et al.,* 2004). Therefore, the effect of hypoxia on promoter activity was tested using the Wayne model (Fig 4.9).

Promoter activity of P_{26} during NRP1 was 7-fold higher than activity of cultures during aerobic growth ($p < 0.05$) (Fig 4.9). Thus P_{26} promoter activity was induced by hypoxia. For upstream region P_{25}, no promoter activity was seen during NRP1 (Fig 4.9). P_{hspx} activity during NRP1 was 185 MU, which is not significantly different from the level of activity of P_{hspx} during aerobic growth (Fig 4.9).

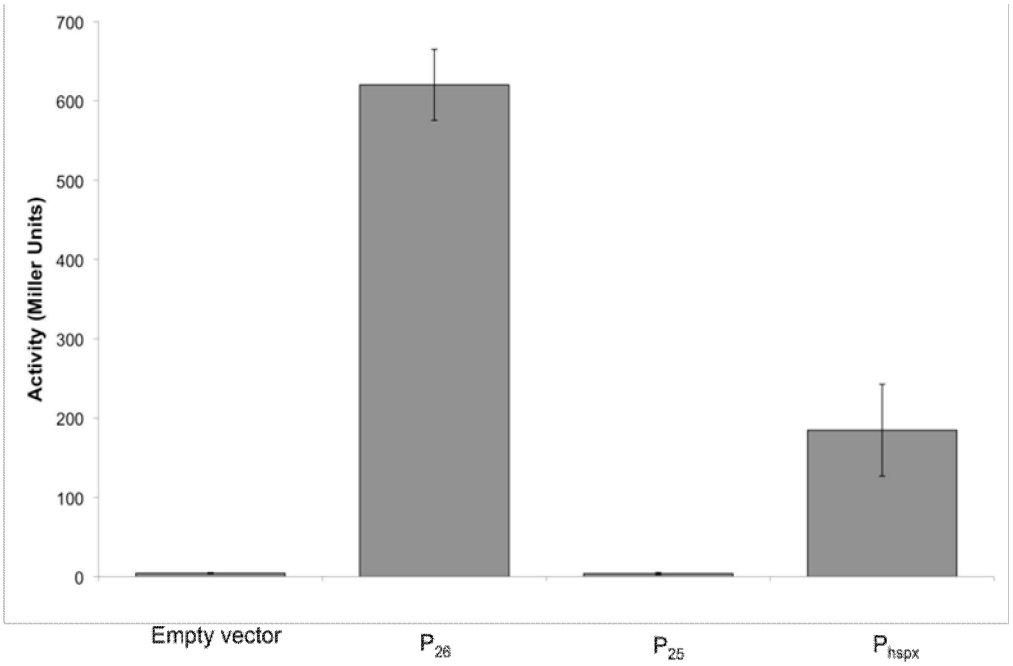

Figure 4.9. Promoter activity of P_{26}, P_{25} and P_{hspx} in the Wayne model in *M. tuberculosis*.

Activity of P_{26}, P_{25} and P_{hspx} in response to hypoxia is shown. The experiment was conducted in Dubos-Tween-Albumin (DTA) medium. Liquid cultures were inoculated to a starting OD_{600} of 0.004 and cell-free extracts were prepared after NRP1 was reached. Methylene blue was used as an indicator of microaerophilic conditions (NRP1). The average activity of three independent transformants assayed in duplicate is given ± standard deviation in Miller Units (measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein).

Expression profiles of promoters in *M. tuberculosis*

A high variation between transformants was observed whilst assessing promoter activity in response to potentially inducing stimuli. This might be due to HspX and DosR expression during stationary phase (Sherman *et al.,* 2001; Voskuil *et al.,* 2004). In order to determine if the promoters are upregulated during growth, promoter activity was measured in relation to optical density of the liquid cultures. Due to the fact that growth rates between transformants may differ, it was decided to use optical density as a measure of growth instead of time. Expression profiles of P_{27}, P_{26}, P_{25} and P_{hspx} were determined by measuring promoter activity during exponential and stationary phase growth. P_{27} was a highly active promoter with a basal level of 700 MU and showed a steady increase in activity during growth (Fig 4.10A). A 8.5-fold increase of activity to 6000 MU was observed when the bacteria were in stationary phase. P_{26} activity was of medium strength and steadily increased in activity with the age of the cultures (Fig 4.11A). Promoter activity in early exponential phase was around 200 MU and increased 22-fold to 4500 MU in stationary phase (Fig 4.11A). P_{25} activity was very low, but steadily increased in activity with growth of the cultures reaching up to a 4-fold increase of activity by stationary phase. (Fig 4.12A). P_{hspx} was a slightly weaker promoter than P_{27} and P_{26}. Its activity was 100 MU during early exponential phase and steadily increased with the age of the culture to a 5-fold high at the end of exponential phase (Fig 4.13A). Activity increased further during early stationary phase and was at its highest (1500 MU) during mid-exponential phase - spanning a 15-fold increase - before it decreased to 400 MU during late stationary phase. An increase of promoter activity in accord with the optical density of the liquid cultures was observed with all promoters tested. This shows that their activity is dependent on growth. Since DosR as well as Rv2627c, Rv2626c, Rv2625c and *hspX* are expressed during stationary phase (Voskuil *et al.*, 2004), it is probable that the increase of promoter activity is due to phosphorylated, active DosR within the cells. Alternatively, the increase in promoter activity with the age of the culture could be an artefact due to continual build up of LacZ from basal activity of the promoters over time. To exclude the idea of LacZ accumulation due to a low turnover rate of the protein, an expression profile of a promoter that is not controlled by DosR

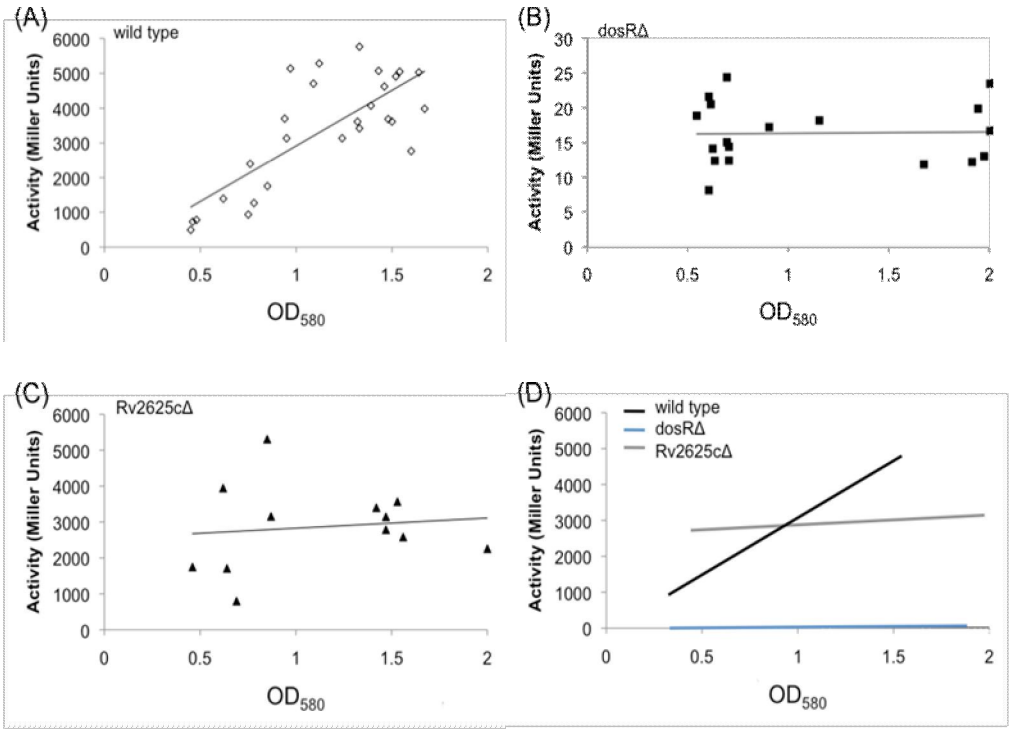

Figure 4.10. Promoter activity of P_{27} during growth in *M. tuberculosis.*

Promoter activity in relation to OD_{580} is shown in (A) wild type; (B) the *dosR*Δ strain; and (C) the Rv2625cΔ strain. An overlay of all three is shown in (D). Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Linear trendlines are indicated. The background activity of pSM128 was 8 ± 3 MU.

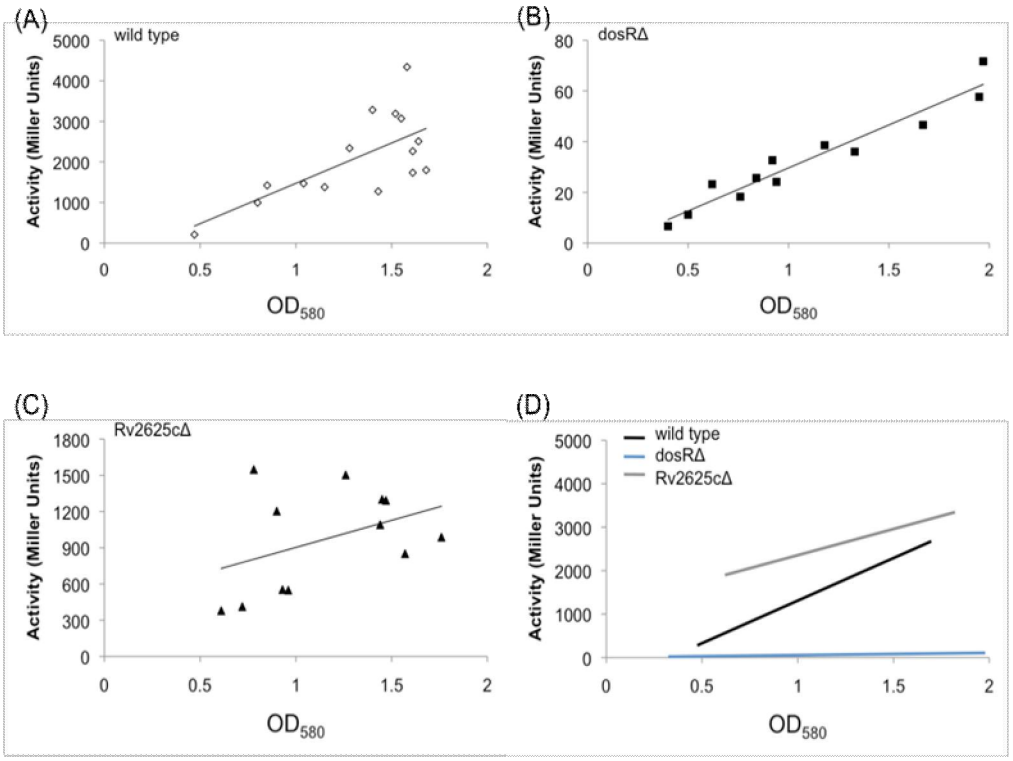

Figure 4.11. Promoter activity of P_{26} during growth in *M. tuberculosis.*

Promoter activity in relation to OD_{580} is shown in (A) wild type; (B) the *dosR*Δ strain; and (C) the Rv2625cΔ strain. An overlay of all three is shown in (D). Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Linear trendlines are indicated. The background activity of pSM128 was 8 ± 3 MU.

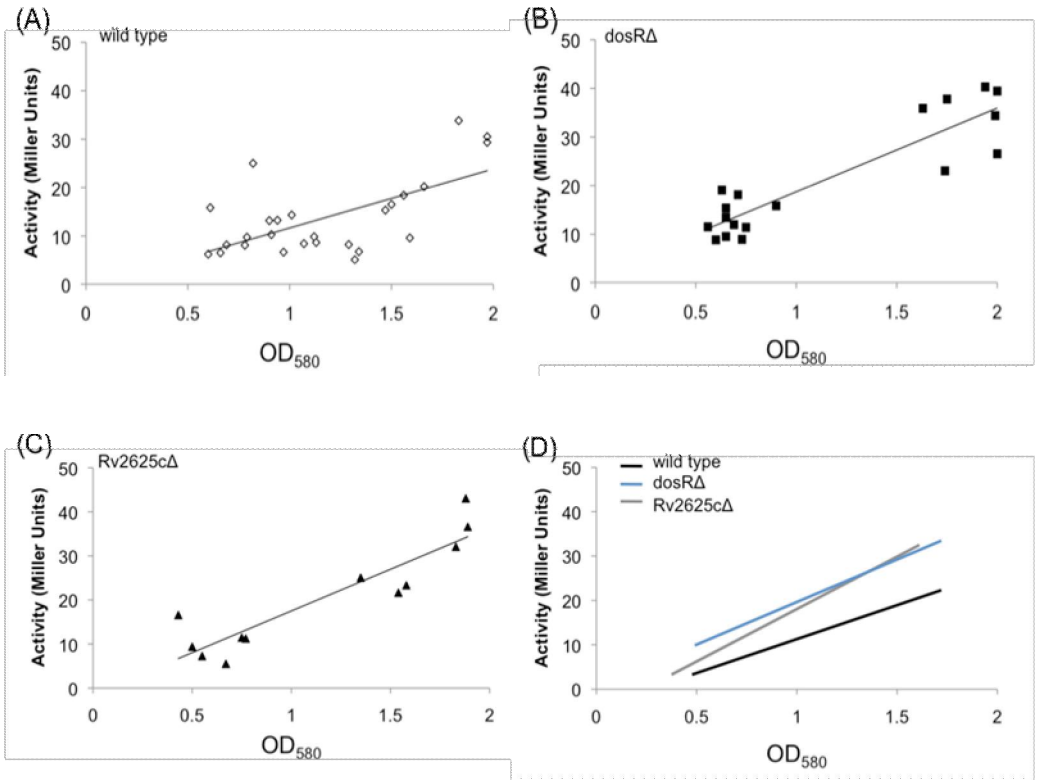

Figure 4.12. Promoter activity of P_{25} during growth in *M. tuberculosis.*

Promoter activity in relation to OD_{580} is shown in (A) wild type; (B) the *dosR*Δ strain; and (C) the Rv2625cΔ strain. An overlay of all three is shown in (D). Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Linear trendlines are indicated. The background activity of pSM128 was 8 ± 3 MU.

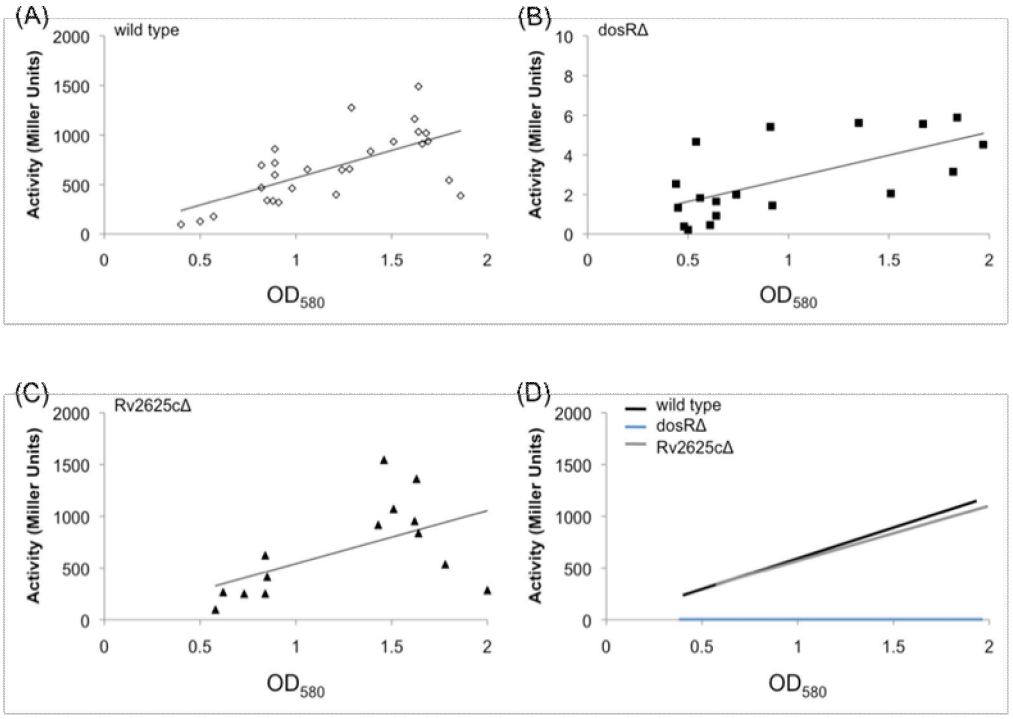

Figure 4.13. Promoter activity of P_{hspx} during growth in *M. tuberculosis.*

Promoter activity in relation to OD_{580} is shown in (A) wild type, (B) the *dosR*Δ strain and (C) the Rv2625cΔ strain. An overlay of all three is shown in (D). Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Linear trendlines are indicated. The background activity of pSM128 was 8 ± 3 MU.

was created. The promoter of the gene *embA* was chosen because it has previously been characterised and has an activity of around 300 MU in *M. tuberculosis*, which is comparable to the basal levels of activity of P_{27}, P_{26} and P_{hspx} (Amin *et al.*, 2008). Cultures were set up as described for P_{27}, P_{26}, P_{25} and P_{hspx} and the expression profile is depicted in Figure 4.14.

Activity during growth remained constant at 300 MU (Fig 4.14), confirming that LacZ is not accumulated over time. Increased promoter activity of P_{27}, P_{26}, P_{25} and P_{hspx} with the age of the culture was due to an increase of expression of *lacZ*, as opposed to accumulation of LacZ.

Expression profiles in the *M. tuberculosis dosR* mutant strain

In order to investigate if the promoters identified are controlled by DosR, expression profiles of the promoters were characertised in a *dosR* mutant strain (TAME16). In this *dosR*Δ strain, a defined region of 447 bp is deleted in *dosR* (Parish *et al.*, 2003; Parish and Stoker, 2000).

P_{27} activity was almost completely abolished in the *dosR*Δ strain (7-25 MU) and was not activated during growth (Fig 4.10B). This was in stark contrast to its profile in the wild type (Fig 4.10D). This shows that P_{27} activity is completely dependent on DosR. In contrast, P_{26} activity was reduced by several orders of magnitude in the absence of DosR but still demonstrated induction during growth. The promoter had a basal activity of 5 MU which increased to a maximum of 70 MU (Fig 4.11B and D). The fact that overall activity of the promoter was reduced, yet the increase in activity remained, is intriguing. Therefore DosR appears to modulate the strength of this promoter, and another regulator controls the pattern of activity during growth. Activity of P_{25} started at a basal level of around 5 MU and steadily increased with the age of the cultures to 40 MU (Fig 4.12B). This pattern of activity is the same that has been seen in the wild type (Fig 4.12D) and shows that growth dependence of P_{25} is independent of DosR. P_{hspX} activity was also abolished, with no increase in activity during growth. Activity measured ranged from 2-5 MU, which corresponds to the background activity of the empty vector (Fig 4.13B). Thus there was no activity of the promoter of *hspX* in the absence of DosR.

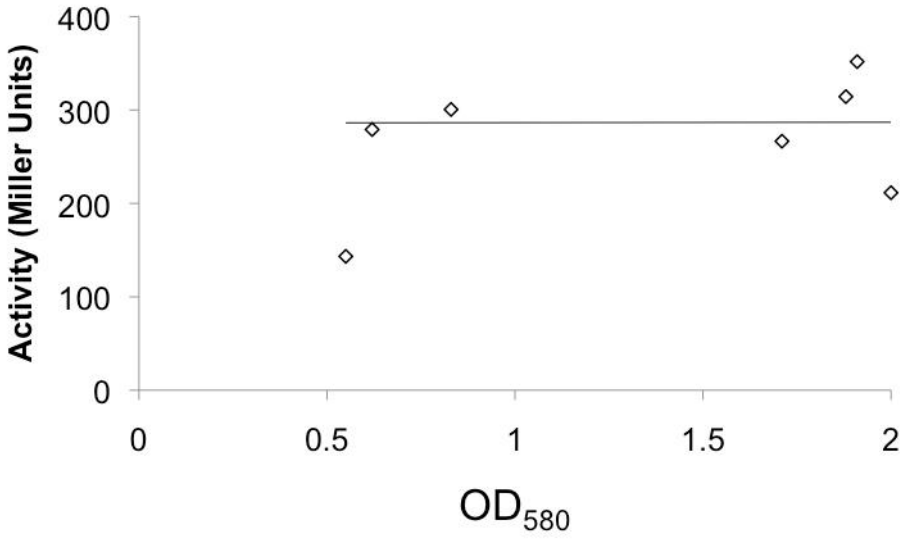

Figure 4.14. Promoter activity of P_{embA} during growth in *M. tuberculosis.*

Promoter activity in relation to OD_{580} is shown. Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. A linear trendline is indicated. The background activity of pSM128 was 7 ± 4 MU.

Expression profiles in the *M. tuberculosis* Rv2625c mutant strain

Activity of P_{27} and P_{hspx} is completely dependent on DosR, whereas activity of P_{26} is only partly DosR-dependent and P_{25} activity appears to be independent of DosR. There must be a regulator other than DosR responsible for the growth dependence of P_{25} and P_{26}. This is interesting in light of the organisation of the Rv2625c region, with Rv2626c and Rv2625c being very close together (Fig 4.4). Unpublished microarray data (Parish and Muttucumaru, unpublished) indicates that Rv2625c could be a protease involved in a regulatory pathway and may autoregulate its expression. It is possible that the protein encoded by Rv2625c is the regulator responsible for the growth dependence of P_{26} and P_{25}.

To investigate the possibility of Rv2625c autoregulation, expression profiles of the promoters were characertised in an Rv2625c mutant strain (NAME3). In this strain a part of the Rv2625c gene has been deleted (unpublished data).

P_{27} activity was affected by the absence of Rv2625c. Even though P_{27} was a highly active promoter and its activity increased with the age of the cultures, activity during mid to late stationary phase was lower than in the wild type ($p < 0.05$) (Fig 4.10C and D). Thus the growth dependence of P_{27} is DosR- as well as Rv2625c-dependent. P_{26} activity was of medium strength in the Rv2625cΔ mutant and increased with the age of the cultures (Fig 4.11C). The pattern of growth dependence and the magnitude of activity during exponential phase (going from 200-1000 MU) were the same as in the wild type (Fig 4.11D). However, once the bacteria entered stationary phase, there appeared to be no further increase in promoter activity, which remained around 1500 MU, compared to 3000-4000 MU in the wild type ($p < 0.05$). This shows that Rv2625c influences P_{26} activity during stationary phase. Overall, these results hint towards a dual regulation of P_{26} by DosR and Rv2625c. In the Rv2625cΔ mutant, P_{25} activity was unaffected and steadily increased from 10 MU to 40 MU as it did in the wild type (Fig 4.12C and D). This shows that the growth dependence of P_{25} is not affected by Rv2625c, and thus Rv2625c does not regulate its own expression through P_{25}. Similarily to P_{25}, P_{hspx} activity was unaffected by the deletion of Rv2625c and continued to increase with the age of the culture, reaching maximum

activity during mid-stationary phase (1500 MU) (Fig 4.13C and D). This confirms that P_{hspx} activity is completely dependent on DosR alone.

Expression profiles in Dubos-Tween-Albumin (DTA) medium

Assaying promoter activity of P_{27}, P_{26} and P_{hspx} in a *dosR* mutant strain has shown that their activity during aerobic growth is regulated by DosR. A recent study showed that expression of DosR is medium-dependent (Malhotra *et al.*, 2009). The study used quantitative RT-PCR (qRT-PCR) to measure aerobic expression of DosR in exponential phase cultures in different media. It was found that in *M. tuberculosis* H37Rv DosR expression is significantly less when grown in DTA medium than DosR expression in 7H9 medium. These two media differ mostly in the nitrogen sources that they contain: 7H9 contains ammonium sulphate and L-glutamic acid whereas DTA contains pancreatic digest of casein and L-asparagine (Appendix 11). From Malhotra's results it may be expected that activity of promoters that are regulated by DosR would be reduced in DTA medium.

To investigate if activity of P_{25} or the DosR-dependent promoters was also medium-dependent, expression profiles of the promoters were determined during growth in DTA medium.

The pattern of activity of P_{27} was the same in both types of medium, with P_{27} activity being dependent on growth phase. However, P_{27} activity during late exponential phase was increased in DTA medium, ranging from 1000-10000 MU when compared to its activity in 7H9 medium ($p < 0.05$) (Fig 4.15A). This increase in strength hints at differences in DosR expression or activity between the different media. P_{26} activity was high and steadily increased in activity with the age of the cultures in DTA. The pattern of growth dependence and the magnitude of activity during exponential phase (going from 200-2000 MU) were similar to that observed in 7H9 (Fig 4.15B). Surprisingly, P_{26} activity was higher in DTA than it was in 7H9 medium during exponential phase. However, once the bacteria entered stationary phase, there appeared to be no further increase in promoter activity, which levelled at around 2000 MU. Thus the type of media used affects P_{26} activity during stationary phase ($p < 0.05$). This is intriguing, as a similar occurrence was observed in the Rv2625c deletion strain. In DTA medium, activity of P_{25} started at a basal level of around 5 MU and steadily increased with the age of the cultures to 14 MU in late stationary phase which is in the range of

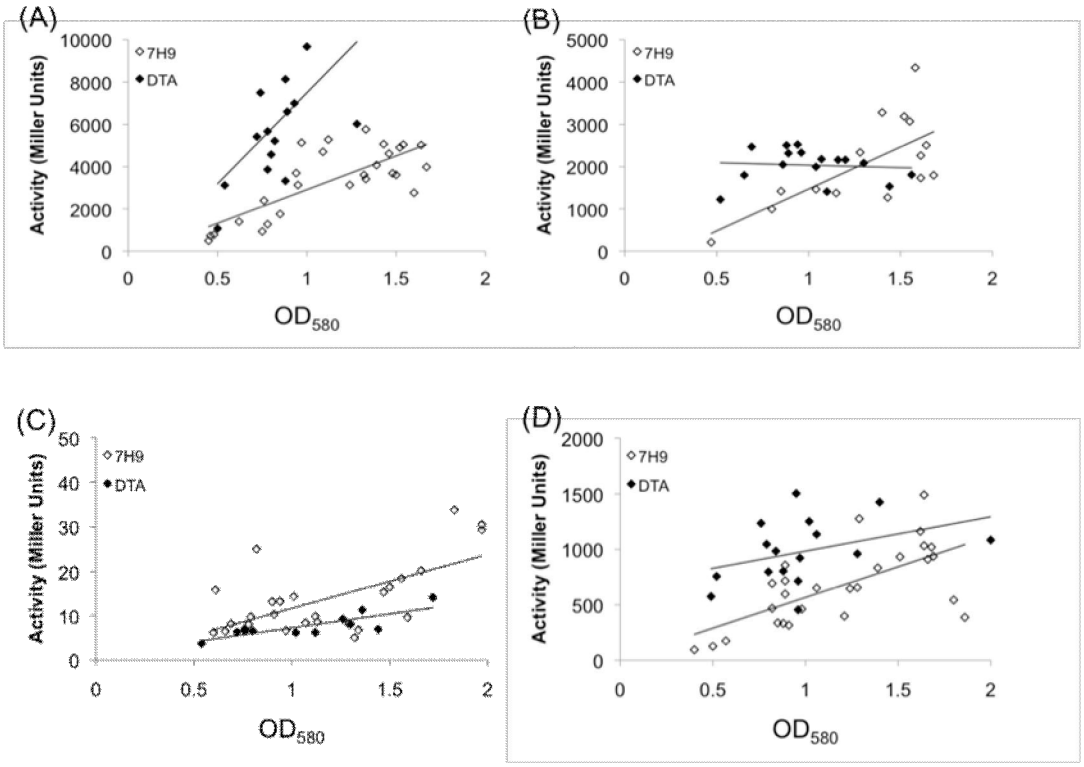

Figure 4.15. Promoter activity in 7H9 and DTA media in *M. tuberculosis.*

Promoter activity of (A) P_{27}, (B) P_{26}, (C) P_{25} and (D) P_{hspx} in relation to OD_{580} is shown. Liquid cultures were grown in 7H9 or DTA medium from a starting OD_{580} of 0.1 up to an OD_{580} of 2. Samples were taken every 4 d and promoter activity was measured. Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (in Miller Units Miller measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Linear trendlines are indicated. The background activity of pSM128 was 8 ± 3 MU.

activity observed in 7H9 medium at this stage of growth (Fig 4.15C). The pattern of activity is the same in both types of medium. Thus P_{25} activity is not medium-dependent. This would be expected, the expression profile in the dosR deletion strain has shown, P_{25} activity to be independent of DosR. In contrast, P_{hspx} displayed the same pattern of expression in both types of media and, similarly to P_{27}, increased in activity during exponential phase growth in DTA medium when compared to 7H9 (Fig 4.15D). Again, this is surprising, since one would have expected its strength to decrease if DosR was downregulated during exponential phase when bacteria are grown in DTA.

Investigating if P_{25} contains a functional promoter

The results of the expression profiles confirmed the dependence of P_{27}, P_{26} and P_{hspx} on DosR and dependence of P_{26} on Rv2625c. P_{25} is independent of DosR and Rv2625c. Even though Rv2625c is a member of the DosR regulon, its upstream region does not contain a DosR motif (in contrast to the promoter regions of Rv2626c, Rv2627c and *hspX* which contain two DosR motifs each) (Park *et al.,* 2003). Rv2625c could have its own promoter in P_{25} or it may be in an operon with Rv2626c and thus co-transcribed from P_{26} (Fig 4.4).

In *M. smegmatis*, the activity of P_{25} was only slightly higher than the background obtained from pSM128 (Fig 4.5). The expression profiles in wild type and the *dosR*Δ and Rv2625cΔ strains of *M. tuberculosis* showed that activity only started to increase to above background levels after mid-stationary phase (Fig 10). Thus there is a possibility that activity observed from P_{25} could be an artefact of inserting a sequence into pSM128.

Sequence analysis of the cloned region revealed a putative -10 element (CTTTAT) immediately upstream of Rv2625c (Fig 4.16A and B). To establish if this was a functional promoter the CTTTAT hexamer was mutated by SDM to CTTTGC (P_{25a}). Another putative -10 element (TCTACT) was found within the last 200 bp of the Rv2626c ORF DNA sequence. This hexamer was mutated to TCTGCC (P_{25b}). The region cloned in P_{25} contains 200 bp of Rv2626c (Fig 4.4).

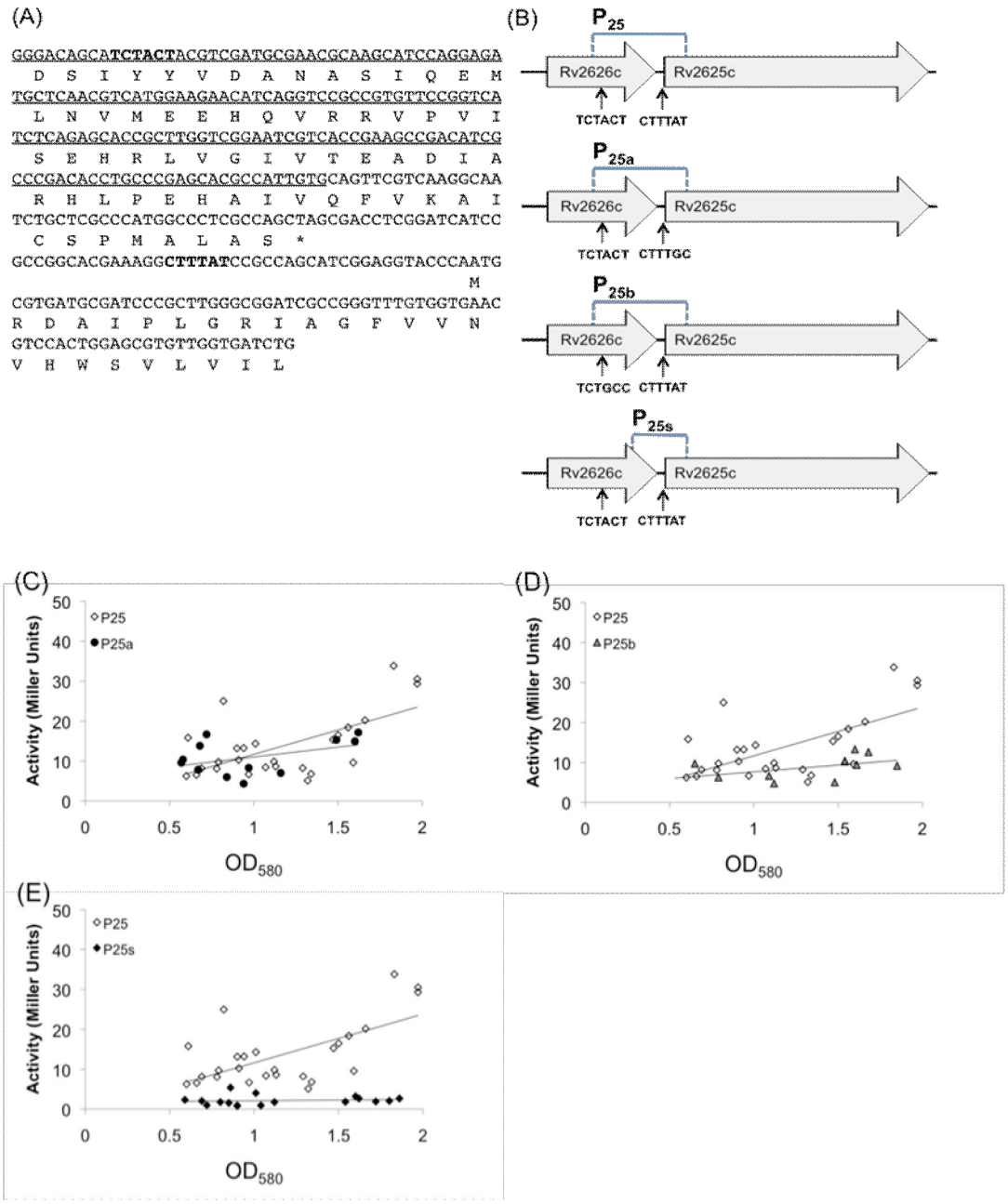

Figure 4.16. P_{25}-variants and their expression profiles in *M. tuberculosis*.

(A) DNA sequence of P_{25}, the Rv2626c ORF and the Rv2625c ORF are shown. Putative -10 elements are in bold and the sequence absent in P_{25s} is underlined. (B) Schematic depiction of P_{25}-variants. Expression profiles of (C) P_{25a}, (D) P_{25b} and (E) P_{25s} in *M. tuberculosis* are shown in relation to that of $P_{25.}$ Three independent transformants were assayed. Each cell-free extract was assayed in duplicate and the average activity (Miller Units measured as amount of O-nitrophenol produced (nmol) over time (min) per mg of protein in the sample) was plotted against OD_{580}. Background of pSM128 was 8 ± 3 MU.

In order to see if removing this sequence affected promoter activity, a shorter construct was made, which included less of the Rv2626c ORF (P_{25s}). Promoter probe plasmids were electroporated into *M. tuberculosis* and expression profiles of the different constructs were determined.

P_{25a} activity ranged from 10 MU to 20 MU up until mid-stationary phase (Fig 4.16C), which is the same range as and not different from P_{25}. Thus the hexamer targeted with SDM is not the promoter controlling Rv2625c expression during exponential and early stationary phase. P_{25b} activity ranged from 10 MU to 13 MU up until late stationary phase (Fig 4.16D), which is lower than the range of P_{25} and also P_{25a}, but this difference was not statistically signficiant. P_{25s} had no activity (1-5 MU) (Fig 4.16E). This reduces the weight of the argument that the P_{25} activity observed in this study merely originated from inserting a DNA sequence into pSM128. Closer examination of the sequence revealed the -10 element targeted in P_{25b} (TCTACT) is missing in this construct (Fig 4.16A and B). The absence of this motif, and other regulatory elements closeby, in P_{25s} could be responsible for the lack of promoter activity.

Analysis of transcripts from the Rv2625c region in *M. tuberculosis*

Expression profiles of the promoters of the genes of the Rv2625c region have shown that the promoter of Rv2626c (P_{26}) was dependent on Rv2625c and DosR. P_{26} activity was reduced during stationary phase growth in the Rv2625cΔ strain when compared to the wild type. If Rv2626c expression is dependent on Rv2625c during stationary phase growth one would expect the Rv2626c transcript levels to be reduced in the Rv2625cΔ strain when compared to wild type and there would be no difference in the amount of transcript present in exponential and stationary phase cultures.

P_{25} was confirmed to contain a functional promoter within the Rv2626c ORF. However, this promoter is very weak and the intergenic region between Rv2626c and Rv2625c is very short, thus P_{25} might not be the only promoter that controls Rv2625c expression. Rv2625c could be in an operon with Rv2626c and thus co-transcribed from P_{26}. Investigating the types of transcripts present from the Rv2627c-Rv2625c region might give insight into which promoter Rv2625c is expressed from during aerobic growth.

To determine the types of transcripts present in the wild type and the Rv2625Δ strain during aerobic growth from Rv2627c, Rv2626c and Rv2625c, RT-PCR was carried out using the primers listed in the appendix. The primers were designed to amplify the 5' ends of each ORF as well as the junction between the genes (Fig 4.17A). RNA was extracted from mid-exponential and late stationary phase (when the promoters are most active) aerobically grown liquid cultures of wild type and the Rv2625cΔ strain of *M. tuberculosis* and cDNA prepared and used as template for PCR.

To ensure that input amount of cDNA into the PCR was the same for each sample, primers that amplify the mycobacterial gene *sigA* were used as a control. cDNA prepared from wild type exponential phase and stationary phase had the same intensity (Fig 4.17B), thus input amount of template was the same for all samples. As expected, transcripts containing the 5' end of this housekeeping gene were present under all conditions tested.

Bands of the expected size to correspond to the 5' end of Rv2726c, the Rv2627c-Rv2626c junction, the 5' end of Rv2626c and the Rv2626c-Rv2625c junction were amplified from both the wild type and the Rv2625cΔ strain cDNA during all phases of aerobic growth (Fig 4.17A and B). This shows that the transcripts containing these regions were present under all conditions tested. Transcripts containing the 5' end of Rv2625c were only detected in the wild type and not in the Rv2625cΔ strain, which is as expected since the deletion mutant does not contain the 5' end of Rv2625c (Fig 4.17A and B).

Quantification of transcripts from the Rv2625c region in *M. tuberculosis*

To quantify transcript amounts during the different phases of aerobic growth, limiting dilution RT-PCR was carried out. In a PCR, the relationship between input template and PCR product is non-linear. To measure differences in mRNA level more accurately, 4-fold serial dilutions of the cDNA were prepared in order to be able to titrate an end-point where no PCR product was observed. In such a scenario, increased levels of mRNA would produce a higher titer of cDNA, and therefore more dilutions are needed to reach the end-point. The number of dilutions taken to reach end-points for each region amplified by PCR are listed in Table 4.2. To ensure that input amount of cDNA into the PCR *sigA* containing transcripts were

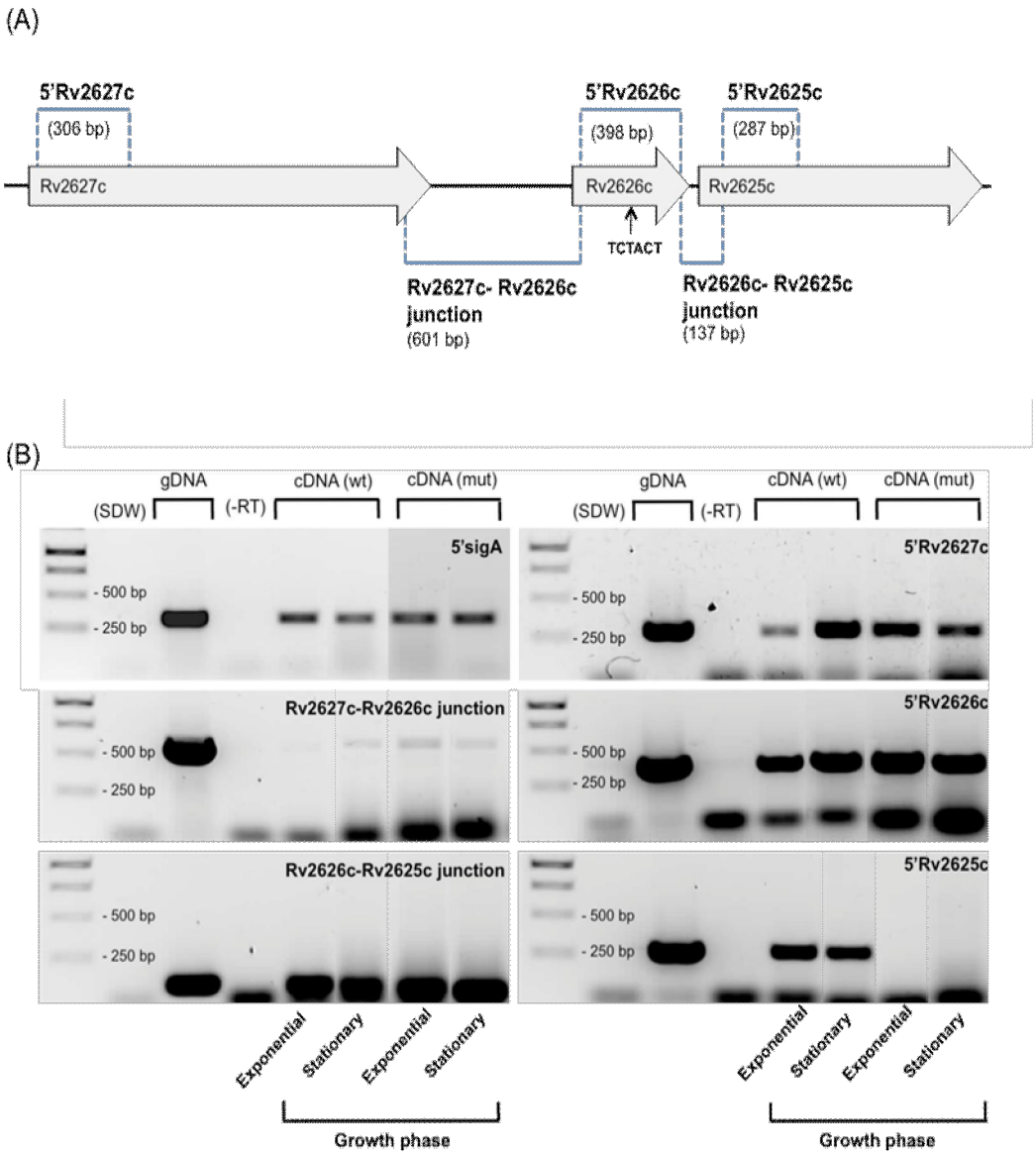

Figure 4.17. Depiction of the Rv2625c region and results from RT-PCR analysis of cDNA from *M. tuberculosis*.

(A) schematic representation of 5' ends and junction regions amplified for RT-PCR is shown. (B) Undiluted RT-PCR products. The type of template which was genomic DNA (gDNA) or complementary DNA (cDNA) is indicated at the top of the wells. Reactions containing water (SDW) or no reverse transcriptase (-) were used as negative controls. cDNA was prepared from RNA extracted during exponential or stationary phase growth of wild type (wt) or a Rv2625cΔ strain (mut) of *M. tuberculosis*. The part of the Rv2625c region amplified is shown in the top right corner.

quantified as a control. cDNA containing the 5' end of *sigA* reached end-points at the same dilution (Table 4.2). This verifies that equal amounts of cDNA were used in each PCR reaction. In the wild type, there was approximately 4-fold more transcript of 5' Rv2627c during stationary phase than there was during exponential phase (Table 4.2). This is as expected, since P_{27} activity peaks during stationary phase growth. In contrast to this, amount of transcripts was the same during both exponential and stationary phase growth in the Rv2625cΔ strain, which would indicate that Rv2627c is not upregulated during statonary phase in the absence of Rv2625c, which corroborates the promoter activity data.

In both the wild type and the Rv2625cΔ strain the amount of transcript containing the Rv2627c-Rv2626c junction was low and was the same during both growth phases (Table 4.2). Since both Rv2627c and Rv2626c have their own strong promoters that control their transcription, one would not expect them to be co-transcribed. The few transcripts which contain the junction region could originate from a low rate of Rv2627c read-through.

The amount of transcripts containing the 5' end of Rv2626c was increased during stationary phase in the wild type (Table 4.2). This is consistent with the observation that P_{26} activity is at its maximal level during stationary phase, confirming that the gene is transcribed throughout aerobic growth and upregulated during stationary phase. In the Rv2625cΔ strain, transcript levels remained unchanged between the two growth phases. This is in accordance with the promoter data of Rv2626c, where P_{26} activity did not increase any further during stationary phase growth in the absence of Rv2625c. It is important to note that there appears to be more transcript from this region in the absence of Rv2625c, since the end-points from the wild type are higher. This is surprising, since from the P_{26} promoter activity data this gene is less induced during stationary phase in the Rv2625cΔ strain. Thus, one would have expected there to be less transcript of this region from Rv2625cΔ stationary phase cDNA than from wild type cDNA.

In the wild type, as well as in the Rv2625cΔ strain, transcript levels of the Rv2626c-Rv2625c junction remained the same during exponential or stationary growth phase. This would not be expected if the 5' end of Rv2625c and the junction between Rv2626c and Rv2625c were on the same transcript (i.e. Rv2625c was co-transcribed with Rv2626c), as in this

Transcript	Wild type cDNA (wt)		Rv2625cΔ cDNA (mut)	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
5' sigA	16	16	16	16
5' Rv2627c	64	256	64	64
Rv2627c-Rv2626c junction	16	16	16	16
5'Rv2626c	16	256	1024	1024
Rv2626c-Rv2625c junction	64	64	64	64
5' Rv2625c	64	64	0	0

Table 4.2. Relative amount of transcripts from the Rv2627c-Rv2625c region in *M. tuberculosis.*

End-points of limiting dilution RT-PCR analysis for each region amplified from wild type and Rv2625cΔ strain cDNA are shown. cDNA was prepared from RNA extracted during exponential or stationary phase and serial 4-fold dilutions were prepared before the PCR.

case the amount of transcript would increase during stationary phase as seen for the 5' Rv2626c transcript. Thus, Rv2626c and Rv2625c can be co-transcribed from the promoter of Rv2626c.

Transcript levels containing the 5' end of Rv2625c, were the same in both exponential and stationary phase liquid cultures in wild type (Table 4.2). The fact that there is no apparent change in the amount of transcript suggests that either Rv2625c is not upregulated during stationary phase or that the change is too small to be picked up on by this semi-quantitative method.

4.3 Discussion

One of the objectives of this project was to examine a variety of conditions that have been shown to induce specific genes and identify the responsible promoters. The genes selected for this study were Rv2627c, Rv2626c, Rv2625c and *hspX*. The objective was to identify the promoter regions of these genes and determine whether differential expression occurs in response to standing culture conditions, treatment with ethanol or DETA/NO or during hypoxia. Plasmids containing the upstream regions of these genes were created and promoter activity was assessed by measuring expression of the reporter gene *lacZ*. The promoter of Rv2625c was characterised in further detail and transcripts of the Rv2625c region were analysed.

4.3.1 Promoter activity of P_{26}, P_{25} and P_{hspx} in *M. smegmatis* in response to inducing stimuli

In *M. smegmatis*, no induction of the *M. tuberculosis* promoters was observed. The lack of induction is surprising since the *M. smegmatis* homologue of the regulator, DevR, has a high amino acid identity with its *M. tuberculosis* counterpart, and conservation extends to the DNA binding regions. It is possible that DevR binds to the DosR motifs of the promoters - resulting in weak promoter activity - but the intracellular level of DevR in *M. smegmatis* might not be comparable to that of DosR in *M. tuberculosis* under the conditions used. Alternatively, the lack of induction may be attributable to differences in upstream signalling, which in *M. tuberculosis* is mediated by two sensor kinases (DosS and DosT). *M. smegmatis* possesses one histidine kinase that interacts with DevR, (DevS), with 66% amino acid identity to DosS, which also spans the site of

autophosphorylation (Mayuri *et al.,* 2002). However, unlike DosS, it does not possess a transmembrane domain and is localised in the cytosol (Mayuri *et al.,* 2002). A recent analysis of the redox properties of the heme iron in *M. smegmatis* DevS and amino acid alignments have actually shown it to be more related to the *M. tuberculosis* DosT rather than DosS (Lee *et al.*, 2008c).

When trying to induce P_{26}, P_{25} and P_{hspx} by subjecting the bacteria to hypoxia, promoter activity was found to be repressed. Normally one would set up the Wayne model cultures at a starting OD_{580} of 0.004 in *M. tuberculosis*. In this study, *M. smegmatis* liquid cultures were set up with a starting OD_{600} of 0.04. However, according to the indicator dye methylene blue, microaerophilic and anaerobic conditions were still reached and therefore promoters were exposed to hypoxic conditions. The Wayne model is based on slow adaptation to reducing oxygen levels (Dick *et al.*, 1998; Wayne and Hayes, 1996), and so there is therefore a possibility that the higher inoculum of bacteria led to a fast reduction of the oxygen tension and, whilst not leading to cell death (no activity would have been detected), the change in dynamics of the adaptation may explain the apparent repression of P_{26}, P_{25} and P_{hspx} activity.

4.3.2 Promoter activity of P_{27}, P_{26}, P_{25} and P_{hspx} in *M. tuberculosis* in response to stress stimuli

Out of the three promoters tested, only P_{26} showed inducible activity after exposure to standing culture conditions, ethanol and hypoxia (9-, 6- and 7-fold respectively). The induction ratio in response to standing culture was higher than previously reported (Kendall *et al.,* 2004b), whilst the induction during hypoxia was less than expected (Bacon *et al.,* 2004; Park *et al.,* 2003; Sherman *et al.,* 2001; Voskuil *et al.,* 2004). No induction of P_{25} and P_{hspx} was observed in response to any of the stress stimuli tested. This was unexpected, since the genes have been shown to be upregulated 8- to 25-fold by DETA/NO treatment (Voskuil *et al*., 2003) and 2- to 83-fold under standing culture conditions (Kendall *et al*., 2004b) in previous microarray studies. Lack of induction by ethanol treatment may have been explained by the possibility that there were no increased levels of phosporylated, active DosR in the cell, despite a 70-fold upregulation of *dosR* after exposure to this stimuli (Kendall *et al*., 2004b), i.e. the upregulation of *dosR* would not

necessarily result in induction of the DosR regulon. However, P_{26} activity was ethanol inducible, thus differences in inducibility may been attributed to differences in the DosR motifs of these promoters. Lack of induction of P_{25} might be attributed to the fact that there is no DosR motif upstream of Rv2625c. However, presence of the motif is not always reflective of how strongly the gene is induced since some of the upstream regions of genes in the DosR regulon do not have a motif, and DosR motifs have been found in front of some genes that are not part of the DosR regulon (Park *et al.,* 2003). Another possibility is that P_{25} does not actually contain the only promoter of Rv2625c - despite the presence of a putative -10 element - and Rv2625c is in an operon with Rv2626c or P_{25} activity is not directly regulated by DosR. Failure to induce P_{hspx} was unexpected because *hspX* has been shown to be upregulated 25-fold in response to NO and 83-fold in response to standing (Kendall *et al.,* 2004b ; Ohno *et al.,* 2003; Voskuil *et al.,* 2003). However, variation between transformants in this study may have been responsible for the low or apparent lack of induction of P_{26} and P_{hspx}. An explanation for the high variation in the absence of a stimulus, may be that in some transformants the promoters were already active when the stimuli were applied (since the growth phase that the cultures were in was not determined) before application of stimuli, and thus lead to a high standard deviation within the results. A previous study has shown minor induction of *hspX* following a 10 min centrifugation (Kendall *et al.* 2004). This may be due to bacteria becoming densely packed during centrifugation, resulting in a change in oxygen concentration. The protocol used for the preparation of cell-free extracts includes two 10 min centrifugations. Thus promoter activity could have been induced simply by the preparation of cell-free extracts. However, it is important to note that in the study by Kendall 2004, only a minor induction was observed; and in the data presented here all samples were prepared in the same way, so that comparatively this effect should not have a major impact on the results observed, as in theory further (full) induction should be observed in response to the other stress stimuli. Furthermore, minor induction of promoter activity by centrifugation would only explain that there was activity observed in the abense of an inducing stimulus, and would not account for the high variation seen in the “unstimulated” cultures. Another explanation for the high variation in promoter activity in the absence of a stimulus in certain cultures could be the growth phase that the cultures were in at the

time the cell-free extracts were prepared. DosR is expressed during stationary phase and previous studies have shown *hspX* to be the dominant protein component of stationary phase cultures in *M. tuberculosis* as well as *M. bovis* BCG (Cunningham and Spreadbury, 1998; Sherman *et al.*, 2001; Voskuil *et al.*, 2004; Yuan *et al.*, 1996). In order to avoid trying to induce the promoters when they are already active, it was decided to monitor the activity of the promoters during growth to create an expression profile. Most other studies on DosR and HspX expression in stationary phase do not state the OD_{580} of the culture when *dosR* and *hspX* were upregulated but state the age of the culture in days instead (Cunningham and Spreadbury, 1998; Sherman *et al.*, 2001; Voskuil *et al.*, 2004; Yuan *et al.*, 1996). Since culture conditions can affect growth dynamics, it was decided to monitor activity in relation to the optical density instead of time, since the former had the potential to be a more accurate way of determining the growth phase of the bacteria.

Promoter activity of P_{27}, P_{26}, P_{25} and P_{hspx} in *M. tuberculosis* during aerobic growth

In *M. tuberculosis* wild type, P_{27}, P_{26}, P_{25} and P_{hspx} are all growth phase-dependent and increased in activity as they approached stationary phase. This finding was further supported by RT-PCR in which transcript levels of Rv2627c and Rv2626c were increased during stationary phase. These results concur with other studies in the literature, which have shown these genes to be upregulated during stationary phase, such as an early protein study by Yuan and colleagues conducted in 1996 and a microarray study conducted by Voskuil and colleagues in 2004 (Voskuil *et al.*, 2004; Yuan *et al.*, 1996). Yuan and colleagues demonstrated that little expression of *hspX* occurred in *M. tuberculosis* grown with constant aeration, and that the protein was absent in culture lysates of OD_{650} 0.4 or 0.7 but present in lysates from cultures harvested following the end of exponential phase growth (Yuan *et al.,* 1996). A microarray study by Voskuil and colleagues showed DosR expression increased 2-fold after 14 days of growth (when stationary phase was reached) and induction of *hspX*, Rv2627c, Rv2626c and Rv2625c peaked after 14-24 days of growth (Voskuil *et al.*, 2004). The finding that P_{hspx} is active during early exponential phase growth, as demonstrated by its expression profile in wild type *M. tuberculosis*, contradicts other findings in the literature (Purkayastha *et al.*, 2002).

Purkayastha and colleagues found that the *M. tuberculosis hspX* promoter was inactive in aerated shaking cultures in BCG (Purkayastha *et al.,* 2002). However, this discrepancy may perhaps be attributed to strain differences between *M. tuberculosis* H37Rv and *M. bovis* BCG.

Previous microarray studies have shown hypoxic induction of the DosR regulon to be abolished in a *dosR* mutant strain (Park *et al.,* 2003) and unpublished microarray data has implicated Rv2625c in regulation of expression of other members of the DosR regulon (Parish and Muttucumaru, unpublished). Expression profiles of the promoters in a *dosR*Δ and Rv2625cΔ strain have shown that activity P_{hspx} is regulated solely by DosR and independent of Rv2625c. In contrast to this, P_{27} and P_{26} activity was only partly dependent on DosR, and, in addition, there may be another regulator involved in controlling its activity. This regulator may be Rv2625c because P_{27} and P_{26} activity was affected by deletion of Rv2625c during stationary phase. This implies that Rv2627c and Rv2626c are dependent on Rv2625c and that Rv2625c is responsible for P_{27} and P_{26} activity in the absence of DosR. These results throw an interesting light on the role of Rv2625c in the adaptation to stationary phase. The fact that transcripts containing the 5' ends of Rv2627c or Rv2626c appear to be present in equal amounts during exponential and stationary phase and that more P_{26} transcripts appear to be present in the Rv2625cΔ strain than in the wild type is intriguing. However, this may be explained by the limiting dilution RT-PCR only being a semi-quantitative method or differences in mRNA stability or reporter protein turnover. Performing qRT-PCR instead might give a more accurate measure of gene expression.

P_{25} activity was not affected by the absence of DosR. This might be explained by the fact that there is no DosR motif directly upstream of Rv2625c and that P_{25} activity is just not (solely) regulated by DosR during aerobic growth. The P_{25} region was shown to contain a functional promoter, and so the theory that the weak activity of P_{25} is due to the fact that it does not contain the Rv2625c promoter region is undermined. P_{25} activity was not affected by the deletion of Rv2625c, excluding the possibility of autoregulation of Rv2625c as indicated by unpublished microarray data (Parish and Muttucumaru, unpublished).

A recent study using qRT-PCR has reported *dosR* expression to be medium-dependent in *M. tuberculosis* H37Rv. *DosR* expression was found to be reduced during exponential aerobic growth in DTA medium (Malhotra *et al.*, 2009), although a Western blot carried out in the same study showed that there was no difference in DosR expression. Activity of the DosR-dependent promoters P_{27} and P_{hspX} was not found to be reduced during exponential phase in DTA medium, thus supporting the view that *dosR* expression is not lowered when bacteria are grown in this type of medium. The increased activity of P_{27}, P_{26} and P_{hspX} during exponential phase growth in DTA medium may be a result of increased DosR activity in this type of medium. Activity of other transcriptional regulators might also be affected by DTA medium, since P_{26} expression was found to be lower than has been previously observed in 7H9 during stationary phase growth.

4.3.3 Regulation of Rv2625c through P_{25}

From the expression profiles it is unclear if P_{25} contains the promoter regulating Rv2625c, because activity measured from P_{25} was very weak. Removal of a short sequence within the ORF of Rv2626c completely abolished promoter activity and thus verified that this region contains a promoter element. These results throw an interesting light on genomic organisation of Rv2626c and Rv2625c, since the transcriptional start site of Rv2625c must be within the Rv2626c ORF. This is in contradiction with the annotation of the Rv2626c ORF and the predicted Rv2625c ORF on the TubercuList database. The true translational start site of Rv2625c could be mapped in order to determine how far down it is from the transcriptional start site within the Rv2626c ORF. In order to further confirm the possibility that Rv2625c expression is also be regulated by P_{26} one could perform a Northern blot to look at the size of the transcript that contains Rv2625c would also clarify under which conditions P_{26} is the promoter that regulates Rv2625c expression.

The biological relevance of the weakness of the P_{25} promoter is intriguing. It is possible that the Rv2625c protein is only required at very low levels, and this only during stationary phase. However, this would only apply if P_{25} is the sole promoter for Rv2625c. A dual regulation of Rv2625c through its own as well as the Rv2626c promoter could also be possible. This potential complex regulation of Rv2625c could be deciphered by determining which

promoter Rv2625c is expressed from under other conditions that induce the gene.

As yet, the precise function of Rv2625c is unknown. The results presented here support earlier (unpublished) findings that Rv2625c may be involved in gene regulation. One could speculate that Rv2625c, a putative transmembrane bound CBS domain-containing peptidase might affect gene regulation of other genes through cleavage of a transcription factor or regulator of gene expression. It is also possible that Rv2625c somehow interacts with Rv2626c at the cell membrane, since Rv2626c also contains a CBS domain, its C-terminal is prone to proteolytic cleavage and the protein is secreted during standing culture conditions (Sharpe *et al.*, 2008). It would be interesting to elucidate how the Rv2625c protein influences expression of other genes (such as Rv2627c and Rv2626c) and if the protein acts in accord with one ore more proteins through a regulatory cascade.

To conclude, except for P_{26}, none of the promoters identified and characterised in this study were found to be inducible by stress stimuli such as ethanol, DETA/NO treatment or standing culture conditions. However, the promoters were all induced during stationary phase growth in *M. tuberculosis*, and unexpectedly the promoters were already active during exponential phase growth. The fact that promoter activity of P_{27}, P_{26}, P_{25} and P_{hspx} increased gradually with the OD_{580} of the culture has important implications for studying the effect of inducing stimuli on these promoters. If the promoter is already expressed at near maximal level, no further increase in activity would be observed even if an inducing stimulus was applied to the culture. Thus any experiments testing whether a promoter is induced by a certain stimulus should be carried out at a low OD_{580}. Even though P_{27}, P_{26} and P_{hspx} were medium strength/strong and inducible promoters, the fact that they have a high basal level of activity, which additionally increases with growth, rules out their use for direct promoter replacement studies. However, the strength of the promoters makes them useful for an antisense setting i.e. for expression of antisense RNA. Antisense knockdown of a reporter gene or mycobacterial gene of interest during stationary phase could be attempted by expression of an antisense message from these three promoters.

5. A salicylate-inducible promoter

5.1 Introduction

The aim of this project was to characterise and utilise a wide range of endogenous promoters to express mycobacterial genes in a controllable fashion under a variety of conditions, including *in vivo* infection models. To control expression of a gene of interest during an infection model, the regulator of the promoter must be of low toxicity and be able to breach biological barriers in order to reach the bacteria (i.e. have good bioavailability). Attempts to identify endogenous antibiotic-inducible promoters have proved fruitless. Therefore, it was decided to search for promoters responding to a different type of inducer, equally suitable for *in vivo* use.

5.1.1 Salicylate-inducible promoter systems

Salicylate-inducible genes can be found in a variety of organisms such as *Arabidopsis thaliana*, *E. coli* and *Bacillus megaterium* (Cohen *et al.*, 1993; Koornneef *et al.*, 2008; Shaw *et al.*, 1997). An acetyl salicylic acid (ASA) inducible promoter system originating from *Pseudomonas putida* has previously been shown to be suitable for *in vivo* use in a mouse model (Royo *et al.*, 2007). The system is based on the fusion of two regulatory circuits (Cebolla *et al.*, 2001). One of the circuits originates from inducible naphthalene degradation in response to salicylate in *P. putida.* The circuit is controlled by the LysR-type regulator NahR and its cognate promoter P_{sal} and originates from the NAH7 plasmid (Cebolla *et al.*, 2001; Schell *et al.*, 1990; Schell and Poser, 1989; Yen and Serdar, 1988). NahR has been extensively studied and its inducer and promoter binding sites have been identified (Bosch *et al.*, 2000; Cebolla *et al.*, 1997, 2001; Park and Madsen, 2004). The second circuit originates from an inducible toluene-xylene catabolic pathway and consists of a salicylate-responsive mutant of the AraC family regulator XylS2 and its cognate promoter P_m, which is carried on the TOL plasmid (Cebolla *et al.*, 2001; Gallegos *et al.*, 1997; Harayama *et al.*, 1989). These two circuits were combined into one tightly controlled transcriptional regulatory cascade which is highly inducible (up to 150-fold) *in vitro* (Cebolla *et al.*, 1997, 2001; Royo *et al.*, 2007; Suarez *et al.*, 1997).

The salicylate-inducible system was used to control expression of cytosine deaminase (*codA*) in *Salmonella enterica*. Cytosine deaminase produces the antitumor agent 5-fluorouracil from hydrolysing 5-fluorocytosine. Mice that were infected with the *Salmonella* (which preferentially colonise tumour tissues) were given salicylate. Successful induction of the system *in vivo* was achieved, as determined by reduced tumour growth in mice that were exposed to salicylate (Royo *et al.*, 2007). Their results clearly demonstrate that a salicylate-inducible promoter system can be functional during an infection model, making salicylate an attractive inducing stimulus. Acetyl salicylic acid (ASA or aspirin) is a well characterised compound which has had its pharmacokinetics studied extensively, for example it is quickly converted to salicylate, which has a half-life of 2-4 hours (Hennekens, 2002; Weissmann, 1991; Yin *et al.*, 1998).

5.1.2 Salicylate and mycobacteria

In plants such as *Arabidposis thaliana*, salicylate is involved in defence against invading pathogens (Liu *et al.*, 2010). In Gram-negative bacteria and in Gram-positive bacteria, salicylate can induce a multiple antibiotic resistant phenotype (Gustafson *et al.*, 1999; Price *et al.*, 2000; Rosner, 1985). In *M. tuberculosis*, salicylate induces a low level of resistance, namely up to two times the MIC of para-amino-salicylate (PAS), streptomycin or ethambutol and, to a lesser extent, to isoniazid and rifampicin. Although the mechanism of induction of multidrug resistance has not been determined, there are parallels with the salicylate induced MarR-AB system from *E. coli*, because *M. tuberculosis* has a MarA homologue (Rv1931) (Schaller *et al.*, 2002). In *E. coli*, salicylate induces the transcriptional regulator MarR, which activates expression of MarA and MarB, which in turn evoke induction of a regulon that includes efflux pumps (Alekshun and Levy, 1997; Pomposiello *et al.*, 2001). Alternatively, salicylate could reduce cell wall permeability and thus account for the low level of drug resistance seen in *M. tuberculosis* (Schaller *et al.*, 2002).

In *M. tuberculosis*, salicylate exposure affected the expression of 58 genes, as determined by a microarray study (Denkin *et al.*, 2005). The majority of these (30 genes) were downregulated and included proteins involved in transcription, translation and synthesis of ATP or fatty acids. This indicates that salicylate treatment results in a general reduction of transcriptional and

translational activities, as well as changes in energy metabolism. Of the 22 genes that were upregulated, the majority encode proteins predicted to be membrane or transmembrane proteins. The most highly induced genes were Rv0560c (a methyl transferase), Rv0599c (a secreted protein of unknown function) and Rv0188 (a transmembrane protein of unknown function). Upregulation of Rv0560c and Rv0599c was verified by quantitative real-time PCR and found to be 208- and 13-fold respectively. None of the *mar* operon homologues of *M. tuberculosis* were induced, suggesting that the mechanisms of salicylate-induced multidrug resistance differs from *E. coli* (Denkin *et al.*, 2005).

Salicylate and PAS strongly induce a 27-kDa protein encoded by Rv0560c in *M. tuberculosis*, but not in *E. coli*, *M. smegmatis* or *M. bovis* BCG (Sun *et al.*, 2001). Rv0560c is also induced in response to superoxide generators such as menadione, the structurally-related naphtoquinone plumbagin, the peroxisome proliferator gemfibrozil, and its structural relatives fenofibrate and clofibrate (Garbe, 2004). Rv0560c is repressed under normal growth conditions in *M. bovis* BCG and *M. tuberculosis* H37Ra (Sun *et al.*, 2001). Data on whether Rv0560c is upregulated under iron-limiting conditions is unclear. A microarray study showed a 10-fold induction of Rv0560c (confirmed by qRT-PCR) in *M. tuberculosis* grown in iron-limited chemostat cultures (Bacon *et al.*, 2007). However, an earlier microarray study did not identify Rv0560c to be regulated by iron (Rodriguez *et al.*, 2002) and in another study, Rv0560c protein expression was found to be unaffected by iron concentration (Sun *et al.*, 2001). The protein is found to be mainly localised to the cytosol. It shares high similarity with other proteins in *M. tuberculosis* (namely Rv3699, Rv2675c and Rv1377c) as well as homology with methyl transferases from *Streptomyces* species and a methyl transferase involved in ubiquinone biosyntehsis in *E. coli* (Sun *et al.*, 2001). The Rv0560c protein shares similarities in amino acid sequence with *S*-adenosylmethionine-dependent methyltransferases from *Arabidopsis thaliana* (G-mt) and *E. coli* (UbiG) and its genomic location in *M. tuberculosis* is close to genes predicted to be involved in menaquinone synthesis such as *menH* (Rv0558), *menD* (Rv0555) and *menC* (Rv0553) (Garbe, 2004).

5.1.3 Salicylate, iron metabolism and mycobactin synthesis

Several studies have shown that, under conditions of low iron, salicylate accumulates in the supernatant of mycobacteria (Ratledge and Brown, 1972; Ratledge and Winder, 1962). Salicylate is a precursor of the mycobacterial siderophore mycobactin (De Voss *et al.*, 1999; Quadri *et al.*, 1998; Ratledge, 2004). The salicylate synthase (MbtI) generates salicylate from chorismate and is upregulated under conditions of low iron, which explains the accumulation of salicylate in iron-depleted mycobacteria (Harrison *et al.*, 2006; Quadri *et al.*, 1998; Zwahlen *et al.*, 2007). Iron is an indispensable component of many prokaryotic and eukaryotic enzymes. When bacteria encounter conditions of low iron, for example during macrophage infection, they produce iron sequestering siderophores in order to maintain cellular functions (Weinberg, 1999, 2009). An excess of iron can have deleterious effects through generation of oxygen radicals in a process known as Fenton reaction. Therefore, iron acquisition must be a tightly controlled process (Imlay *et al.*, 1988). *M. tuberculosis* produces two types of siderophores or mycobactins, which differ in length and polarity: the hydrophilic carboxymycobactin, which is secreted and the hydrophobic mycobactin, which is cell-associated (Barclay and Ratledge, 1988; Ratledge and Ewing, 1996). Non-pathogenic mycobacteria such as *M. smegmatis* produce exochelin and mycobactins but only small amounts of carboxymycobactin (Ratledge and Ewing, 1996). After sequestration by the mycobactins, iron is transported back into the bacterium through a two component importer of ferri-carboxymycobactin (IrtB-Rv2895c) (Farhana *et al.*, 2008). Intracellular levels of iron are sensed by transcriptional regulators and affect expression of 155 genes, many of which are of unknown function (Calder and Horwitz, 1998; Cole *et al.*, 1998; Rodriguez, 2006; Rodriguez *et al.*, 2002; Wong *et al.*, 1999).

Iron homeostasis is regulated by the transcriptional regulator IdeR in *M. tuberculosis*. IdeR uses iron as a co-factor; in the presence of iron, IdeR binds to iron box motifs upstream of genes and blocks their translation (Chou *et al.*, 2004; Gold *et al.*, 2001; Rodriguez *et al.*, 1999). In the absence of iron, IdeR is inactive and cannot bind to iron box motifs and repression is relieved (Rodriguez *et al.*, 2002). IdeR can function as a positive or negative regulator depending on the location of the iron box in relation to the promoter elements or the transcriptional start site of the genes it regulates

(Rodriguez, 2006). In iron-replete conditions, IdeR positively regulates expression of the iron storage genes *bfrA* and *bfrB*. It negatively regulates expression of genes involved in iron regulated transport, including *irtAB*, or the *mbt* and *mbt-2* gene clusters required for mycobactin synthesis (Gold *et al.*, 2001; Rodriguez *et al.*, 2002).

One of the aims of this study was to construct an inducible promoter system that can be employed to regulate expression of a gene of interest in an *in vivo* model. The *Pseudomonas* P_{sal} system has been shown to work effectively in other Gram-negative bacteria such as *Salmonella* and *Bordetella* (Royo *et al.*, 2007; Suarez *et al.*, 1997). The regulated promoter systems used currently originate from bacteria other than *M. tuberculosis* and do not necessarily work efficiently in *M. tuberculosis*. To ensure that a salicylate-inducible system would work in *M. tuberculosis*, it was decided to use the endogenous systems that are highly upregulated in response to salicylate and identify their promoters.

5.1.4 Potential promoters investigated in this study

The genes selected for this study were Rv0560c and Rv0561c. Rv0560c is predicted to be non-essential and encodes a benzoquinone methyl transferase that may be involved in menaquinone synthesis (Sassetti *et al.*, 2003; Sun *et al.*, 2001). Rv0560c is not expressed during aerobic growth and has been shown to be induced to a lesser extent in intraphagosomal macrophages as well as in a modified Wayne model of anaerobic growth in *M. tuberculosis* (Schnappinger *et al.*, 2003; Starck *et al.*, 2004; Sun *et al.*, 2001). Rv0561c is a gene of unknown function which is postulated to encode an oxidoreductase geranylgeranyl hydrogenase and to be required for survival in primary murine macrophages (Rengarajan *et al.*, 2005). Rv0560c and Rv0561c do not contain an iron box motif in their upstream regions, indicating that their expression is at least not directly regulated by IdeR (Bacon *et al.*, 2007; Rodriguez *et al.*, 2002; Schnappinger *et al.*, 2003; Sun *et al.*, 2001).

5.2 Results

5.2.1 Construction of plasmids

The genomic localisation of Rv0560c suggests that it may be in the same operon as Rv0561c. To determine if the genes had their own promoters or if

they were co-transcribed, the upstream regions of both genes were tested for promoter activity and salicylate inducibility.

For both genes, the regions upstream of the predicted translational start site were cloned into the promoter probe vector pSM128 (Dussurget *et al.*, 1999). The amplified upstream regions of Rv0560c and Rv0561c are referred to as $P_{Rv0560c}$ and $P_{Rv0561c}$ respectively (Fig 5.1).

5.2.2 Promoter activity of $P_{Rv0561c}$ and $P_{Rv0560c}$ in *M. tuberculosis*

M. tuberculosis was transformed with the plasmids and promoter activity was measured in transformants grown to exponential phase (Fig 5.2).

$P_{Rv0561c}$ contains the promoter of Rv0561c as indicated by a medium level of activity ($p < 0.05$) (Fig 5.2A). $P_{Rv0560c}$ contains the promoter of Rv0560c, which was weaker than that of Rv0561c ($p < 0.05$) (Fig 5.2B).

Since Rv0560c is induced in aerobic cultures in response to salicylate (0.5-10 mM) (Sun et al., 2001), it was decided to assess if the promoters of the two genes selected could be induced by a similar range of concentrations (Fig 5.2).

$P_{Rv0561c}$ activity did not change in response to treatment with 0.1-10 mM salicylate (Fig 5.2B). Thus the promoter is not salicylate-inducible. In contrast to this, P_{sal} activity increased up to 3-fold in response to salicylate treatment and maximal induction was reached with 0.5 mM salicylate. Promoter activity did not increase any further when higher concentrations of salicylate were used. This dose-responsive behaviour shows this to be the inducible promoter responsible for Rv0560c upregulation in response to salicylate.

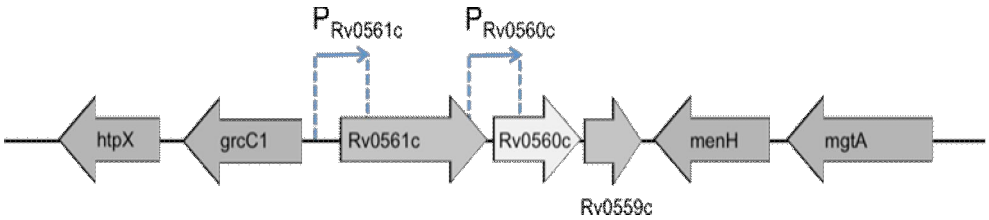

Figure 5.1. The genetic organisation of Rv0561c and Rv0560c in *M. tuberculosis*.

Light grey shaded arrows represent the open reading frames of Rv0560c, Rv0561c and adjacent genes. The upstream regions that were amplified by PCR and cloned into the promoterless expression vector pSM128 are indicated as $P_{Rv0561c}$ (660 bp), and $P_{Rv0560c}$ (690 bp).

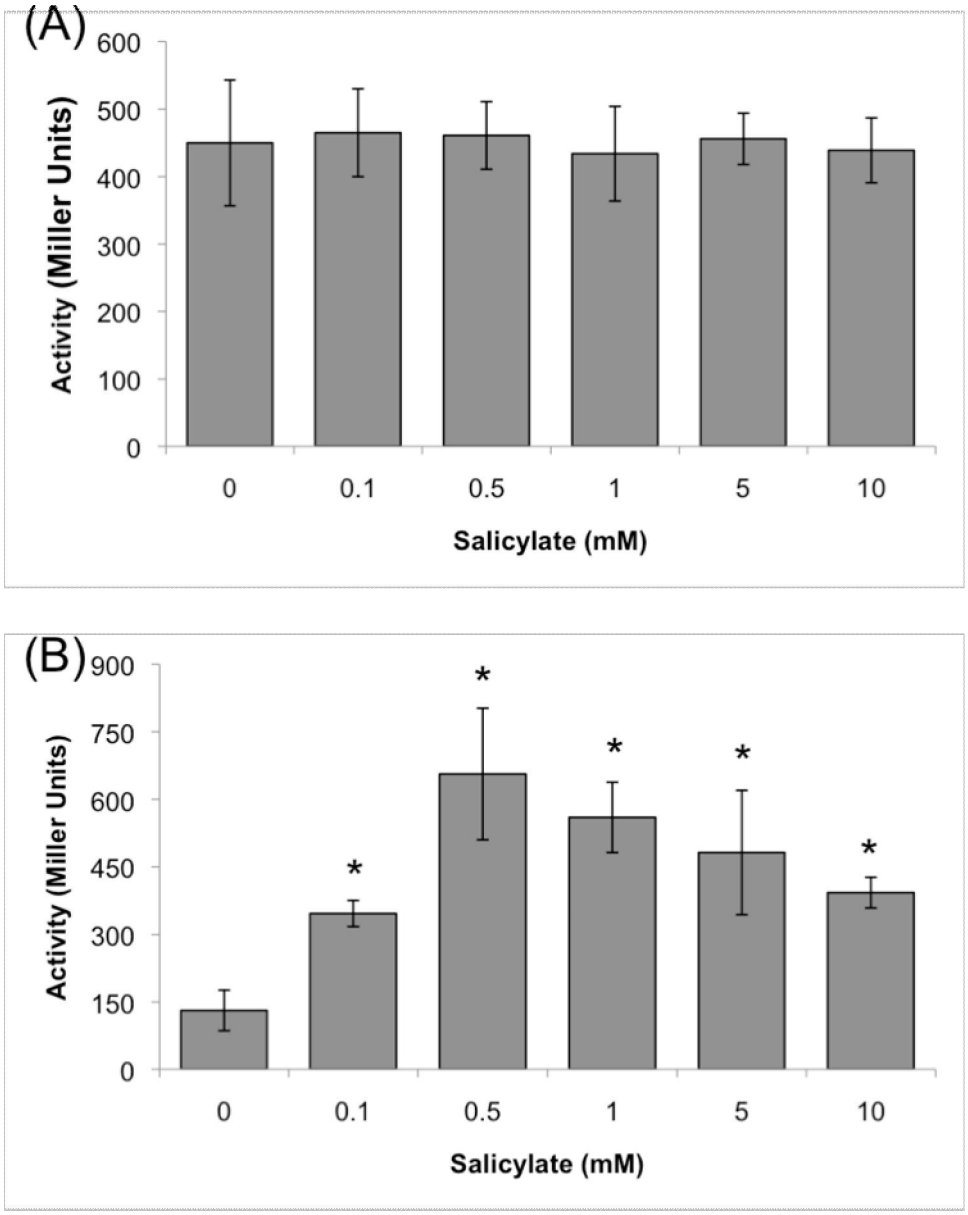

Figure 5.2. **Identification of a salicylate-inducible promoter in *M. tuberculosis.***

Liquid cultures of transformants carrying the (A) $P_{Rv0561c}$ and (B) $P_{Rv0561c}$ plasmids were grown to an OD_{580} of 0.6-0.8 (as an indication of mid- to late exponential phase growth) in 7H9 medium in roller bottles. Aliquots (10 mL) of the rolling culture were subjected to treatment with varying concentrations of salicylate for 2 h. Cell-free extracts were prepared from the cultures and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the untreated control is marked by an * ($p < 0.05$).

It was decided to find the lowest salicylate concentration between 0.1 and 1.0 mM that would give the highest induction of the promoter. Thus, $P_{Rv0560c}$ activity was tested in response to a narrower range of concentrations: 0.1-0.75 mM salicylate (Fig 5.3).

In the absence of inducer, background activity of $P_{Rv0560c}$ was 122 MU (Fig 5.3). $P_{Rv0560c}$ activity increased with salicylate concentration up to 0.4 mM (7-fold induction) and then reached a plateau. This shows that $P_{Rv0560c}$ is both sensitive to and dose-responsive to low concentrations of inducer and that robust induction can be achieved with as little as 0.4 mM salicylate. Unless otherwise stated, this was the concentration chosen for any further experiments investigating $P_{Rv0560c}$ activity in *M. tuberculosis*. There was no difference in viability counts between untreated and treated cultures (data not shown), confirming that salicylate was not toxic under these concentrations and at this treatment length.

Salicylate inhibits the growth of *M. tuberculosis*, with a reported MIC of 0.35-1.5 mM (Sun *et al*., 2001). To ensure that growth of *M. tuberculosis* was not seriously impaired by the presence of inducer, aerobic growth was monitored in liquid cultures without and with salicylate added (Fig 5.4).

In the absence of salicylate, wild type *M. tuberculosis* reached stationary phase after 7 d of growth and continued to grow up to OD_{580} = 2 within 10 d (Fig 5.4). In the presence of 0.4 mM salicylate, growth was slower and bacteria reached stationary phase after 15 d of growth and OD_{580} = 2 after 17 d of growth.

Transformants harbouring the $P_{Rv0560c}$ plasmid grew more slowly than the wild type in the absence of inducer (Fig 5.4). This is not surprising, as the antibiotic present in the medium would be expected to slow down growth of the bacteria. In the presence of salicylate, growth was marked by an extended lag phase. However, although the bacteria took about 15 d to reach stationary phase growth, the cells were still viable and growing. Differences in the length of the lag phase between transformants might be due to strain variation. These results show that the concentration of inducer used does not kill the cells, but slows down their growth.

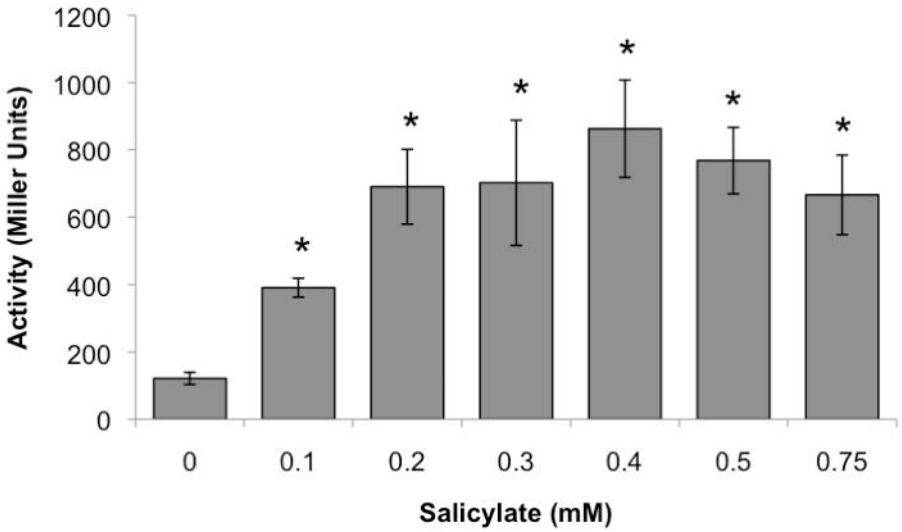

Figure 5.3. Dose-responsive behaviour of $P_{Rv0560c}$ activity in response to salicylate in *M. tuberculosis*.

Liquid cultures of transformants carrying the $P_{Rv0560c}$ plasmid were grown to mid- to late exponential phase in 7H9 medium in roller bottles. Aliquots (10 mL) of the rolling culture were subjected to treatment with varying concentrations of salicylate for 2 h. Cell-free extracts were prepared from the cultures and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the untreated control is marked by an * ($p < 0.05$).

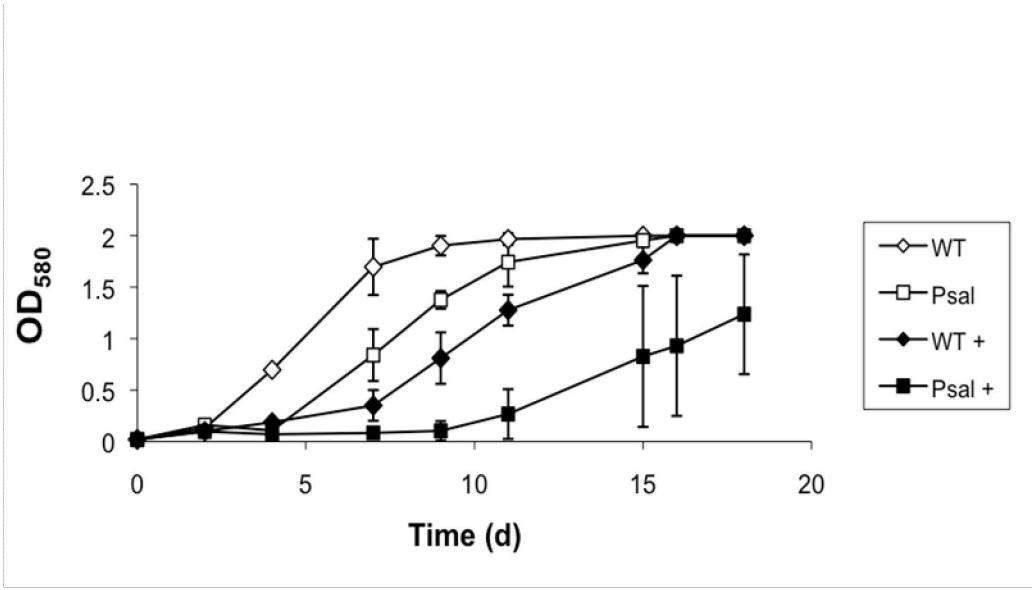

Figure 5.4. **Effect of salicylate on growth of wild-type and $P_{Rv0560c}$ expression plasmid-harbouring *M. tuberculosis*.**

Growth of *M. tuberculosis* wild type in the absence (-) or presence (+) of 0.4 mM salicylate (white and black diamonds respectively) over 18 d. The mean OD_{580} of three independently assayed transformants is shown ± standard deviation in relation to time in days.

5.2.3 $P_{Rv0560c}$ induction kinetics

Robust induction of $P_{Rv0560c}$ activity was achieved after as short a time as 2 h of treatment with 0.4 mM salicylate. It was decided to test if activity of $P_{Rv0560c}$ increased with time of exposure to inducer, and how long after addition of salicylate the promoter remained active for. Induction kinetics of $P_{Rv0560c}$ were investigated by monitoring promoter activity in relation to various lengths of treatment with 0.4 mM salicylate.

$P_{Rv0560c}$ activity tripled from basal level to an activity of 301 MU after as little as 30 min of treatment with the inducer ($p < 0.05$) (Fig 5.5A). Activity increased further with time and a 100-fold increase (10315 MU) was observed after 3 d of treatment (Fig 5.5B). Promoter activity remained induced for up to 35 d after addition of salicylate (Fig 5.5B). These results show that $P_{Rv0560c}$ is induced relatively quickly, but induction to maximal activity is slow. It is an extremely strong promoter with slow induction kinetics: once maximal level of activity is reached it remains stably expressed at a high level for at least 35 d.

$P_{Rv0560c}$ activity was induced 100-fold within 3 d in response to treatment with 0.4 mM salicylate. It was decided to test if induction kinetics were different when lower concentrations of inducer (0.2 mM) were used (Fig 5.6). As seen with the higher concentrations, $P_{Rv0560c}$ activity increased quickly (7-fold after 1 d of treatment), but was slow to reach its highest level of induction (Fig 5.6). When halving the amount of inducer, activity of the promoter was lower and only a 20-fold induction was observed; and that only after 7-14 d of treatment. This shows $P_{Rv0560c}$ to have slower induction kinetics and to be of lower strength when exposed to less inducer. Thus $P_{Rv0560c}$ activity can be modulated depending on inducer concentration and length of treatment.

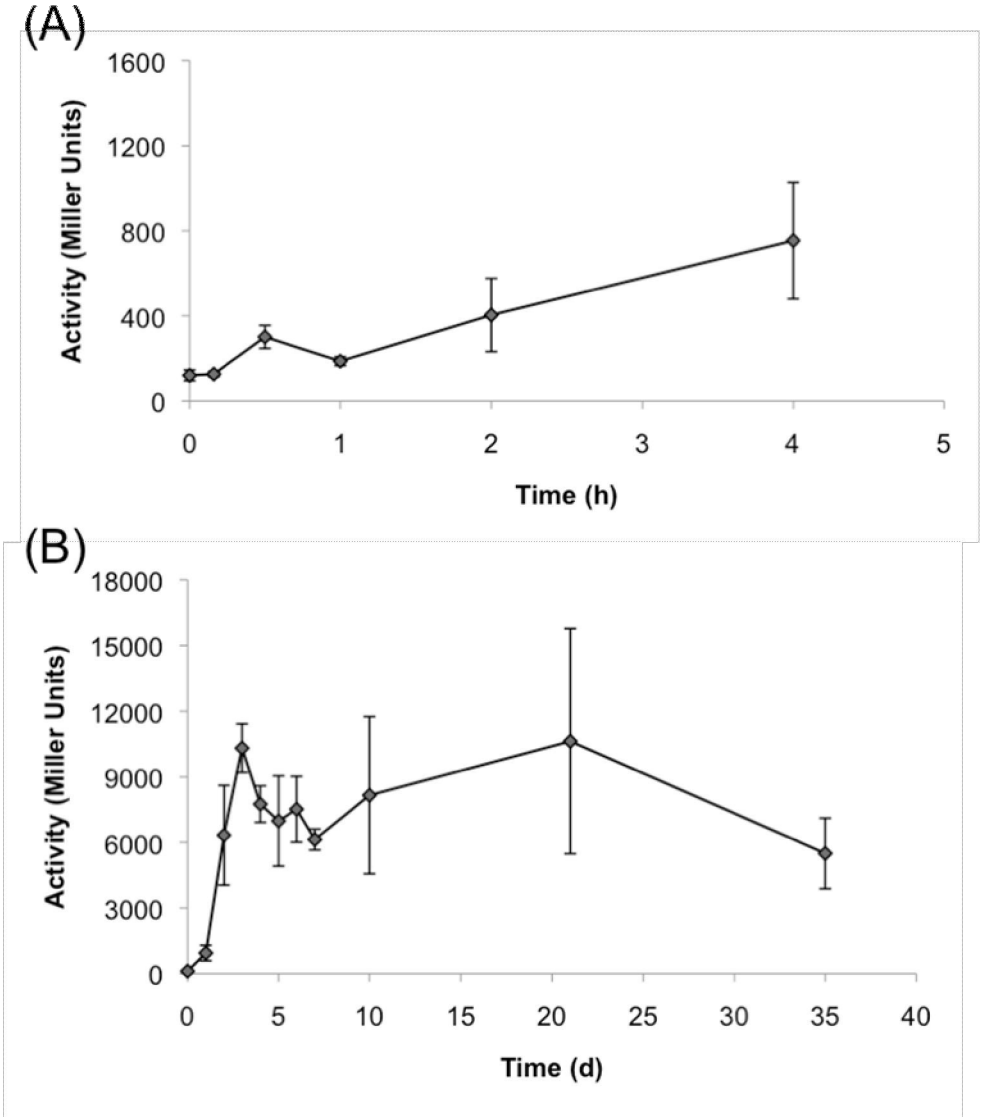

Figure 5.5. **$P_{Rv0560c}$ induction kinetics in response to salicylate.**

Promoter activity of $P_{Rv0560c}$ in *M. tuberculosis* is shown in relation to length of exposure to salicylate. Transformants harbouring the $P_{Rv0560c}$ plasmid were grown to mid- to late exponential phase in liquid cultures in roller bottles. Aliquots (10 mL) were subjected to treatment with 0.4 mM salicylate for a range of times from 10 min up to 35 d. (A) short term induction kinetics and (B) long term induction kinetics. Cell-free extracts were prepared from the cultures after the determined length of salicylate exposure and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU.

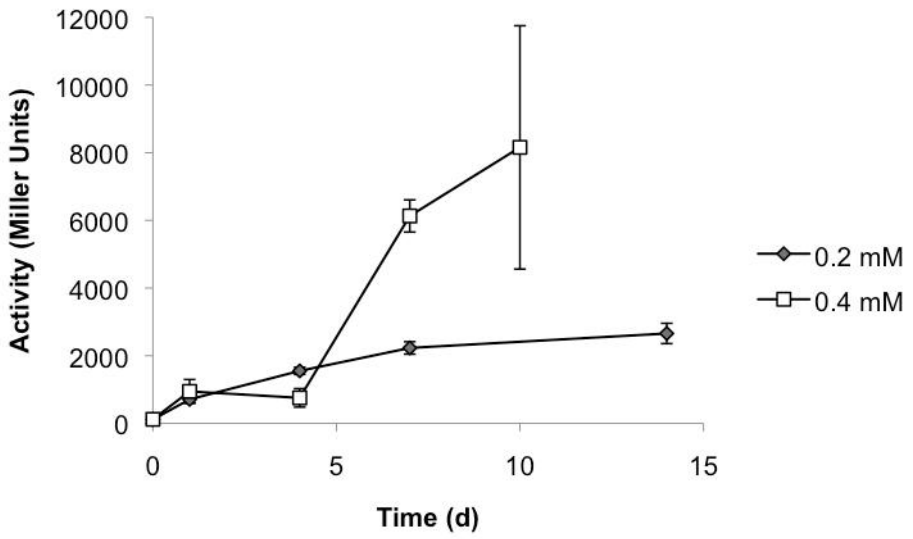

Figure 5.6. $P_{Rv0560c}$ induction kinetics in response to two different concentrations of salicylate in *M. tuberculosis*.

Aliquots (10 mL) from mid-exponential phase cultures grown in roller bottles were subjected to treatment with 0.2 mM or 0.4 mM salicylate for a range of times varying from 1 to 14 d. Cell-free extracts were prepared from the cultures after the determined length of salicylate exposure and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 4 MU.

5.2.4 $P_{Rv0560c}$ activity in response to structural analogues of salicylate

Previous studies have shown that the Rv0560c protein is induced by PAS (a structural analogue of salicylate), superoxide generators such as naphtoquinones (menadione and plumbagin) and fibrates (fenofibrate, clofibrate and gemfibrozil), but not by other structurally-related compounds (aspirin, benzoate or pyrazinoic acid) (Garbe, 2004; Sun *et al.*, 2001). Having a range of compounds with slightly different pharmacokinetic properties available to control expression of an inducible promoter system would be useful. Therfore it was decided to determine if structural analogues of salicylate and other inducers could be used to control the activity of $P_{Rv0560c}$. The structural analogues PAS, aspirin and benzoate, as well as structurally-related compounds menadione, fenofibrate and gemfibrozil were chosen.

$P_{Rv0560c}$ basal activity in the absence of inducer was ~150 MU and unaffected by the DMSO and ethanol solvents used (Fig 5.7). After treatment with salicylate, $P_{Rv0560c}$ activity was induced 64-fold to 9319 MU. $P_{Rv0560c}$ activity was only induced 10-fold by the structural analogues aspirin and PAS (1596 MU and 1484 MU respectively). In contrast, benzoate did not induce $P_{Rv0560c}$. These results show that $P_{Rv0560c}$ induction is specific to a certain structure present in salicylate, aspirin and PAS, but not benzoate. Amongst the fibrates that were tested, fenofibrate evoked a small level of induction (17-fold), whereas gemfibrozil evoked a higher level of induction (41-fold), which is more similar to the effect of salicylate. Menadione repressed $P_{Rv0560c}$ activity 4-fold. These results show that other compounds that are not direct structural analogues of salicylate, can also be used to modulate $P_{Rv0560c}$ activity.

5.2.5 Induction kinetics in response to gemfibrozil

Out of all the alternative compounds tested, gemfibrozil had the most similar effect to salicylate. It was decided to investigate if this compound had the same induction kinetics as salicylate in order to establish if it could be used as alternative inducer.

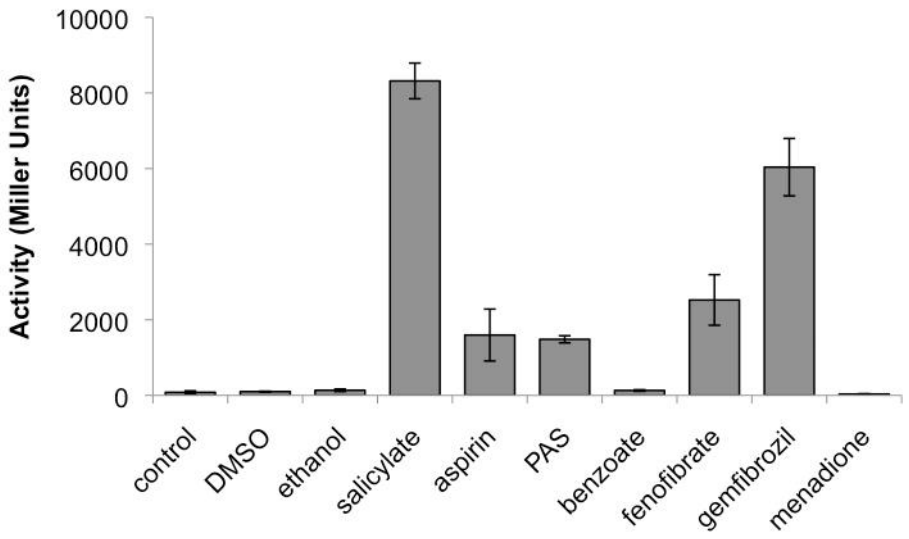

Figure 5.7. $P_{Rv0560c}$ activity in response to structural analogues of salicylate in *M. tuberculosis*.

Transformants harbouring the $P_{Rv0560c}$ plasmid were grown to late exponential phase in liquid cultures grown in roller bottles. Aliquots (10 mL) were subjected to treatment with 0.4 mM of compound for 3 d. Cell-free extracts were prepared from the cultures after treatment and ß-galactosidase activity in the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU. Chemical structures of the compounds used are included as appendix. Except for the untreated or solvent treated controls, a significant difference compared to salicylate-induced activity is marked by an * ($p < 0.05$).

$P_{Rv0560c}$ activity in the absence of an inducing stimulus was found to be 82 MU (Fig 5.8). Upon addition of gemfibrozil, promoter activity increased 20-fold after 2 d, 168-fold after 7 d and peaked at a 184-fold induction after 14 d of treatment. As seen with salicylate activity remained induced at a high level even 21 d after addition of inducer. This shows that despite initial induction kinetics being slower than that of salicylate, $P_{Rv0560c}$ activity was induced to a strength and robustness equal to that of salicylate. This indicates that gemfibrozil could be used an alternative inducer to salicylate.

5.2.6 $P_{Rv0560c}$ OFF kinetics in response to removal of inducer

For every inducible promoter system, it is important to know how long it takes for the promoter activity to return to basal level after removal of the inducer. Consequently it was decided to characterise the OFF kinetics of $P_{Rv0560c}$ in relation to its inducer salicylate.

A profile of the OFF kinetics of $P_{Rv0560c}$ was established. $P_{Rv0560c}$ activity was induced to maximal level by growing *M. tuberculosis* in the presence of salicylate and promoter activity was monitored after removal of salicylate from the growth medium through one wash.

$P_{Rv0560c}$ activity was measured immediately after the wash in salicylate-free medium and was at the maximal level of induction observed previously (Fig 5.9). Activity halved over 3 d to 3849 MU but did not decrease further, indicating that $P_{Rv0560c}$ has very slow off kinetics. A return to basal level was not observed, possibly due to the presence of residual salicylate in the medium or inside the cells.

To ensure removal of all salicylate from the liquid cultures, cells were washed in inducer-free medium multiple times and the effect of the washes on the OFF kinetics of this promoter was assayed.

$P_{Rv0560c}$ activity immediately after the washes was induced to maximal level and was unaffected by the number of washes (Fig 5.10). Promoter activity halved over 7 d of growth in inducer-free medium. Increasing the number of washes carried out to remove salicylate from the growth medium did not allow for promoter activity to return to basal level over the course of 7 d. Thus, residual activity of $P_{Rv0560c}$ above basal level may have be due to intracellular levels of salicylate rather than salicylate left over in the medium after washing.

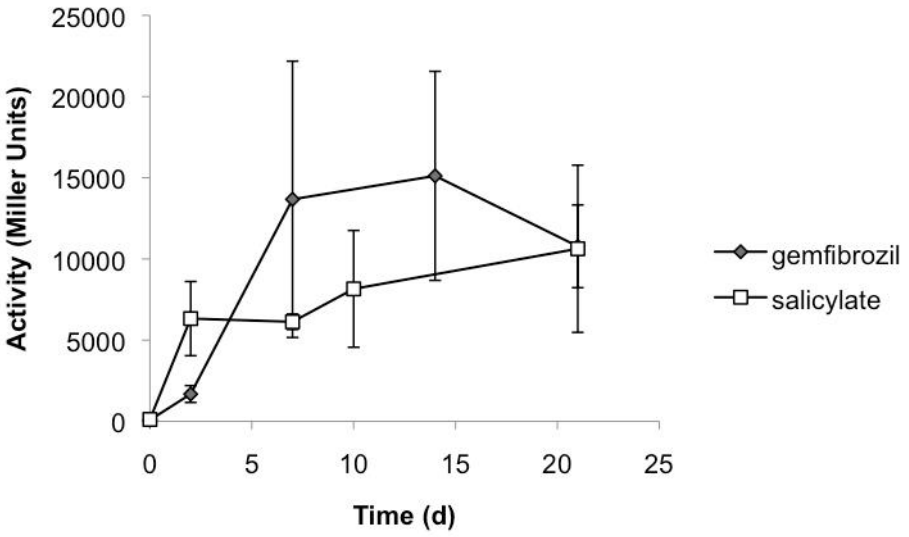

Figure 5.8. $P_{Rv0560c}$ induction kinetics in response to gemfibrozil in *M. tuberculosis*.

Aliquots (10 mL) from late exponential phase cultures grown in roller bottles were subjected to treatment with 0.4 mM gemfibrozil or salicylate for a range of times varying from 2 to 21 d. Cell-free extracts were prepared from the cultures after the treatments and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU.

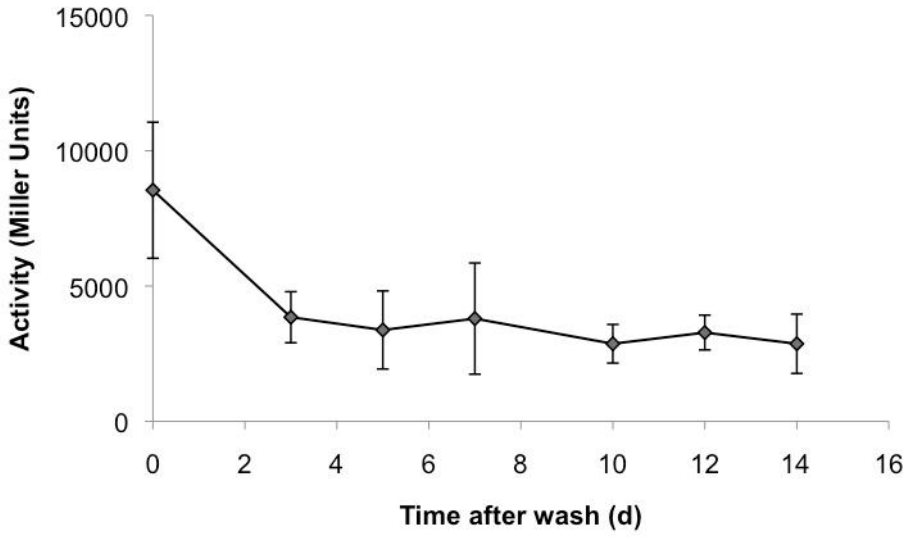

Figure 5.9. $P_{Rv0560c}$ OFF kinetics in salicylate-free medium in *M. tuberculosis*.

Transformants harbouring the $P_{Rv0560c}$ plasmid were grown to mid-exponential phase in liquid cultures in roller bottles with 0.4 mM salicylate. Aliquots (10 mL) were made and cells were washed and resuspended in 10 mL salicylate-free medium. Cell-free extracts were prepared from the cultures immediately (time 0), or after re-incubation in salicylate-free medium and ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU.

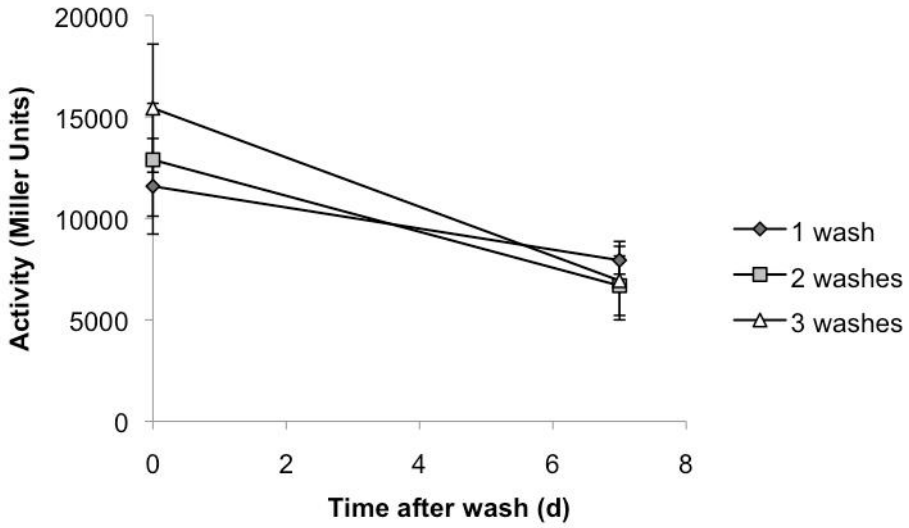

Figure 5.10. $P_{Rv0560c}$ OFF kinetics after multiple washes in salicylate-free medium.

M. tuberculosis transformants carrying the $P_{Rv0560c}$ plasmid were grown to late exponential phase in rolling liquid cultures in the presence of 0.4 mM salicylate. Aliquots (10 mL) were made and cells were washed 1-3x in salicylate-free medium and resuspended in 10 mL salicylate-free medium. Cell-free extracts were prepared from the cultures immediately or after re-incubation for 7 d. ß-galactosidase activity in all samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU.

If the failure of $P_{Rv0560c}$ activity to return to basal level was due to high accumulated intracellular levels of salicylate, one could dilute out the residual salicylate by multiple passages. During each cell division, intracellular levels of salicylate would be halved and thus $P_{Rv0560c}$ activity should decrease with time and bacterial growth. Passages in inducer-free medium were prepared from exponential phase bacteria in which $P_{Rv0560c}$ activity had been induced through growth with salicylate.

Promoter activity decreased 23-fold (to 1302 MU) during the first passage and returned to basal level (200 MU) in the second passage (Fig 5.11A). $P_{Rv0560c}$ activity remained at basal level in the third passage. These results show that dilution of accumulated salicylate inside the cells through multiple passages allows $P_{Rv0560c}$ activity to return to basal level.

5.2.7 Effect of benzoate on $P_{Rv0560c}$ OFF kinetics

Addition of a non-inducing structural analogue of salicylate (benzoate) might allow $P_{Rv0560c}$ activity to return to basal level more quickly after the removal of inducer. Benzoate might displace salicylate from the protein that regulates $P_{Rv0560c}$ activity and thus enable promoter activity to return to its basal level more rapidly.

$P_{Rv0560c}$ was induced by growing bacteria in the presence of salicylate. The cells were washed and resupended in inducer-free medium to which either 0.4 mM or an excess of 4 mM benzoate was added. $P_{Rv0560c}$ activity was induced to maximal level (8542 MU) after growth with salicylate (Fig 5.12). In the presence of 0.4 mM or 4 mM benzoate, activity took 2 d to halve and activity did not decrease any further. Therefore, the addition of benzoate had no effect on $P_{Rv0560c}$ OFF kinetics after removal of inducer through washes in inducer-free medium.

Previous results have shown that dilution of accumulated inducer through passages allowed $P_{Rv0560c}$ activity to return to basal level during the second passage. It was decided to test if $P_{Rv0560c}$ activity would be reduced more quickly in the presence of benzoate.

$P_{Rv0560c}$ activity was induced to maximal level after growth in the presence of inducer (30514 MU) and decreased 24-fold during the first passage (Fig 5.11B). Activity of $P_{Rv0560c}$ reached basal level (220 MU) in the second passage and did not decrease further. These results show that addition of

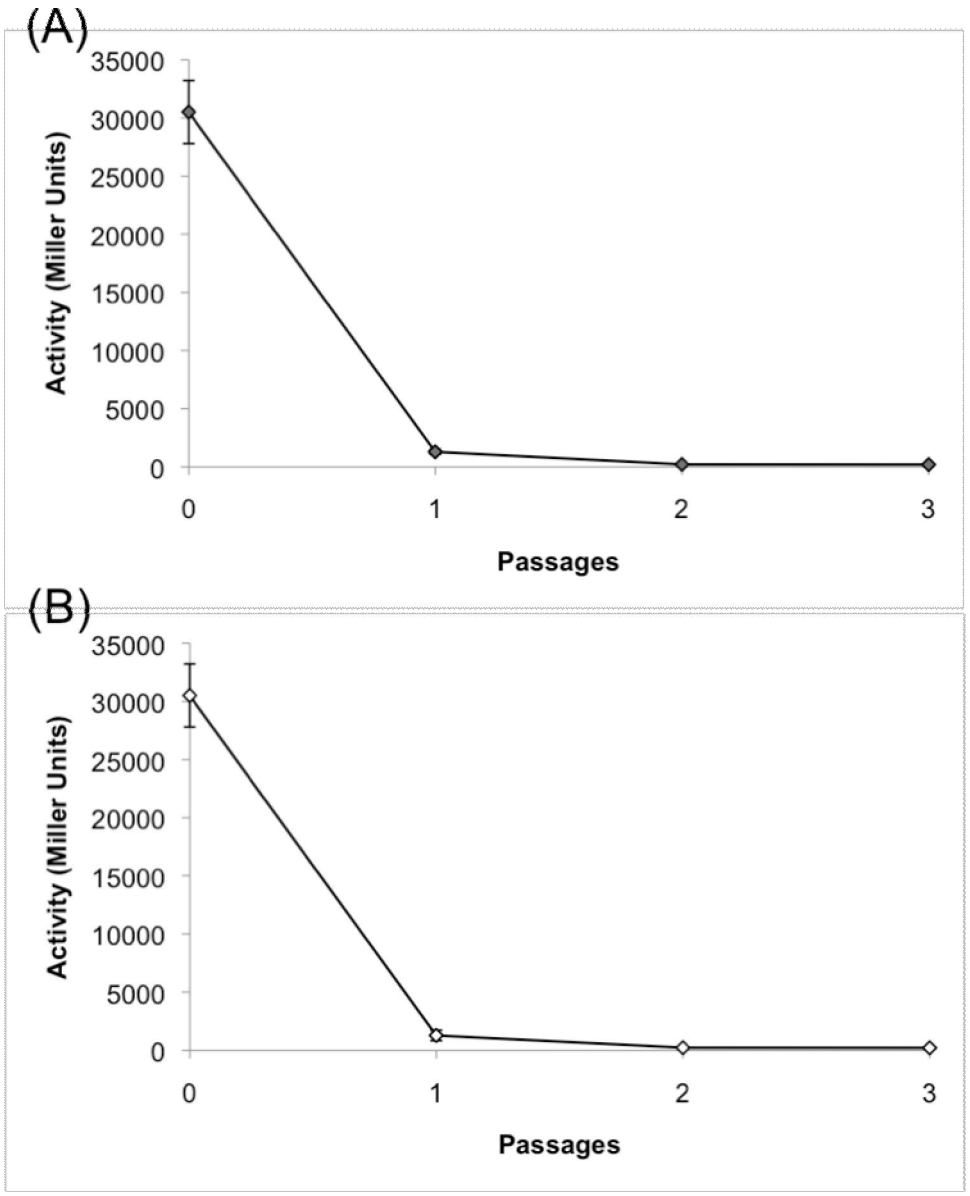

Figure 5.11. $P_{Rv0560c}$ OFF kinetics after passages in salicylate-free medium in *M. tuberculosis*.

Liquid cultures of the transformants harbouring the $P_{Rv0560c}$ plasmid were grown to late exponential phase in roller bottles with 0.4 mM salicylate. The cells were washed and resuspended in (A) salicylate-free medium or (B) medium containing 0.4 mM benzoate. These time 0 cultures were diluted 1/10 to set up a new passage and grown to mid-exponential phase before a new passage was inoculated. Cell-free extracts were prepared during each passage. ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 was 8 ± 3 MU.

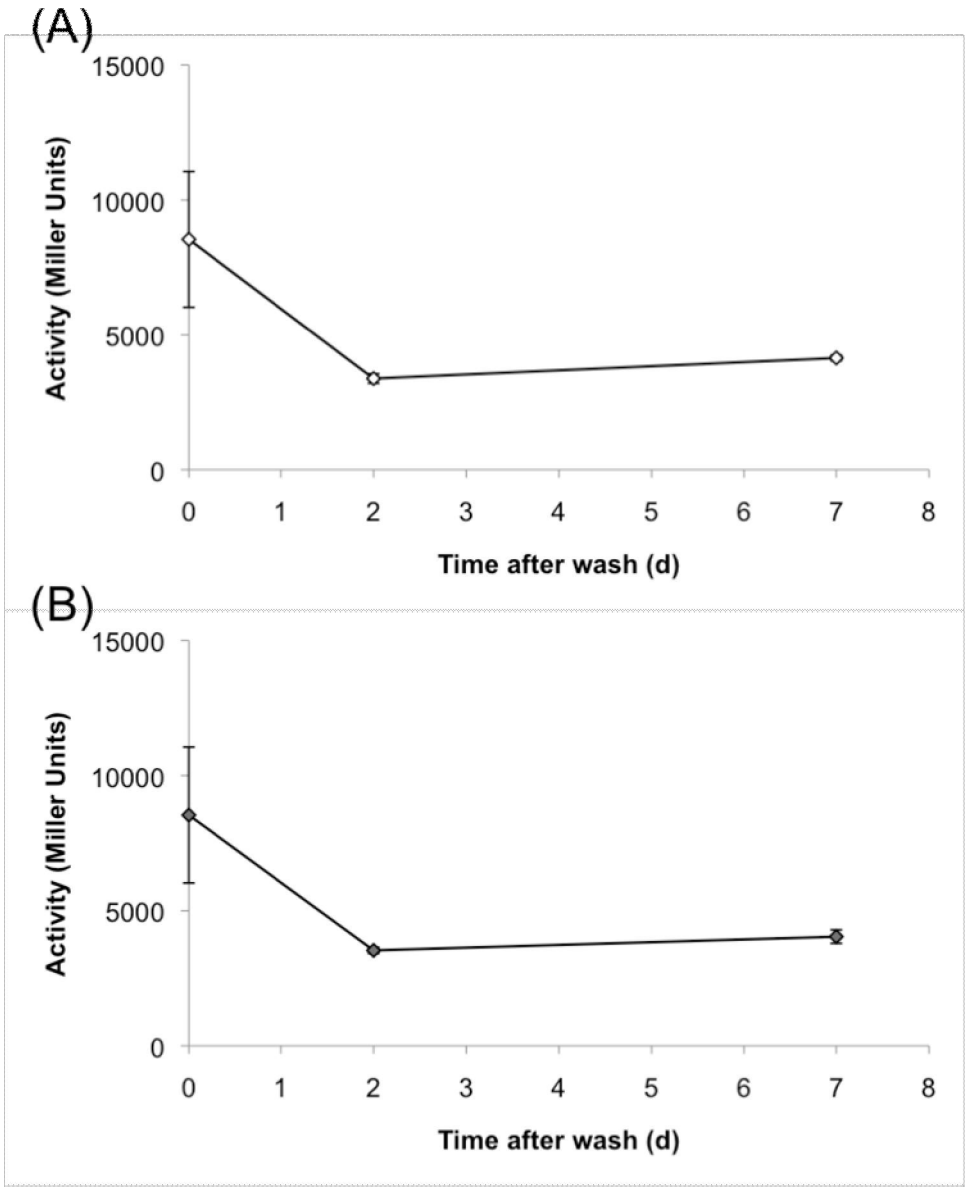

Figure 5.12. $P_{Rv0560c}$ OFF kinetics after one wash in salicylate-free medium in the presence of benzoate.

M. tuberculosis transformants harbouring the $P_{Rv0560c}$ plasmid were grown to mid-exponential phase in liquid cultures in roller bottles with 0.4 mM salicylate. Aliquots (10 mL) were made and cells were washed and resuspended in 10 mL salicylate-free medium. Either (A) 0.4 mM or (B) 4.0 mM benzoate were added to the medium. Cell-free extracts were prepared from the cultures after the wash and addition of benzoate and ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU.

benzoate had no effect on $P_{Rv0560c}$ activity after removal of accumulated inducer through passages.

5.2.8 Induction kinetics of $P_{Rv0560c}$ when linked to unstable LacZ

LacZ is a very stable protein. Whilst $P_{Rv0560c}$ activity could decrease quickly after removal of inducer, a lag due to slow LacZ turnover might prevent this from being observed. Thus it was decided to test both induction and OFF kinetics of $P_{Rv0560c}$ with unstable versions of LacZ. Two protein tags (AANDENYAASV or AANDENYALAA) that would target LacZ for ssrA-mediated degradation were added to *lacZ* in the $P_{Rv0560c}$ promoter probe vector by SDM. These tags have previously been used with GFP and differ in the half-life of the tagged protein (Blokpoel *et al.*, 2003).

To test if induction kinetics of $P_{Rv0560c}$ differed when using the tagged versions of *lacZ,* a time course of promoter activity in relation to various lengths of treatment with 0.4 mM salicylate was established.

Initially, $P_{Rv0560c}$ activity was found to be lower with both tagged versions of LacZ (Fig 5.13). $P_{Rv0560c}$ activity increased 21-fold with ASV tagged LacZ and 12-fold with LAA tagged LacZ 2 d after addition of salicylate. The promoter was induced to maximal activity within 7 d and remained induced over a period of 21 d. Thus, even though the promoter is not induced as quickly as with normal LacZ, induction kinetics and maximal promoter activity are the same between all three versions of LacZ. The slight differences between the tagged versions can be attributed to different half-lives conferred by the tags.

5.2.9 OFF kinetics of $P_{Rv0560c}$ when linked to unstable LacZ

Overall, no significant difference was observed in the induction kinetics of $P_{Rv0560c}$ with the three different versions of LacZ. It was decided to test if this was also true for the OFF kinetics of the promoter or if $P_{Rv0560c}$ activity would return to basal level more quickly with the unstable LacZs.

After growth in the presence of salicylate $P_{Rv0560c}$ activity was induced to maximal level with both versions of tagged LacZ (Fig 5.14). Interestingly, activity from the tagged proteins took more than 2 d to halve, which is slower than what had previously been observed with normal LacZ.

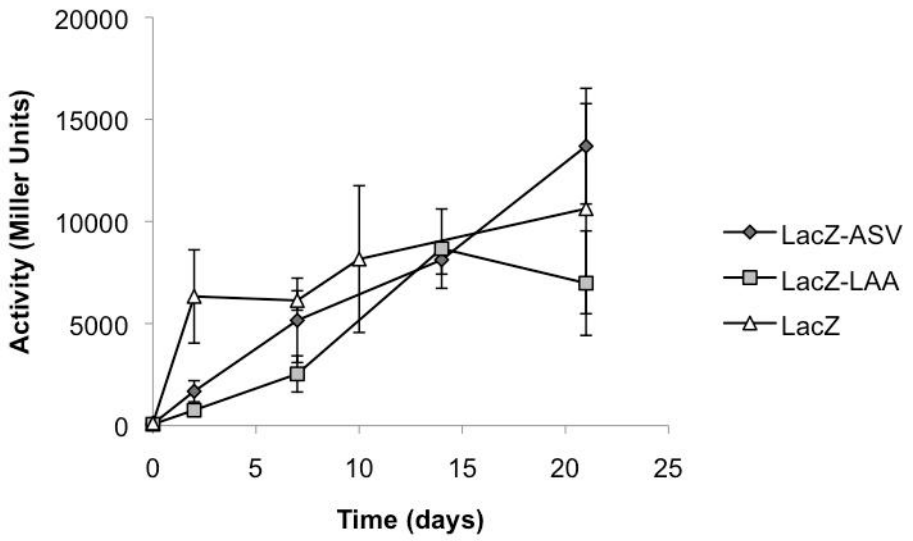

Figure 5.13. Activity of $P_{Rv0560c}$ linked to different versions of LacZ after induction with salicylate.

M. tuberculosis transformants harbouring the $P_{Rv0560c}$ plasmid were grown to mid- to late exponential phase in liquid cultures in roller bottles. Aliquots (10 mL) were subjected to treatment with 0.4 mM salicylate for up to 21 d. Cell-free extracts were prepared from the cultures after the determined length of salicylate exposure and ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU.

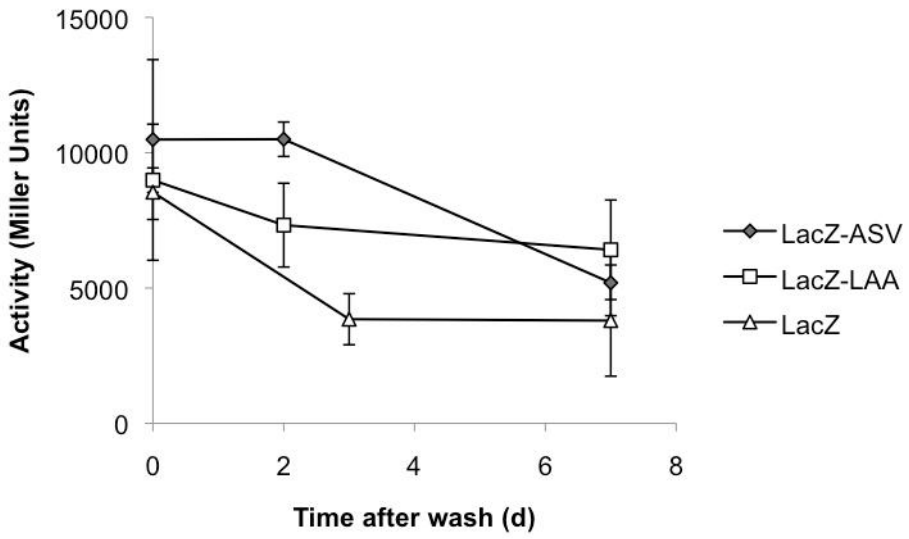

Figure 5.14. OFF kinetics of $P_{Rv0560c}$ linked to different versions of LacZ after removal of inducer from the medium.

M. tuberculosis transformants harbouring the $P_{Rv0560c}$ plasmid with the tagged LacZs were grown to mid-exponential phase in liquid cultures in roller bottles in the presence of 0.4 mM salicylate. Aliquots (10 mL) were made and cells were washed and resuspended in 10 mL salicylate-free medium. Cell-free extracts were prepared from the cultures immediately (time 0), or after re-incubation for up to 14 d. ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU.

However, 7 d after removal of inducer from medium, there was no difference in $P_{Rv0560c}$ activity measured from the different LacZs.

Previous results have shown that when using normal LacZ, $P_{Rv0560c}$ activity reduced 23-fold after one passage in salicylate-free medium. Using unstable LacZ might result in a quicker reduction. Therefore, the activity of induced

$P_{Rv0560c}$ linked to tagged LacZ was assayed throughout the first passage in inducer-free medium. After promoter induction through growth in the presence of salicylate, $P_{Rv0560c}$ activity differed considerably between the types of LacZ used (Fig 5.15). Activity from the tagged LacZs was higher than from normal LacZ. However, $P_{Rv0560c}$ is active at a high level in all three strains at the beginning of the passage. After 2 d of growth, activity in the strain containing normal LacZ was the most reduced. However, by the end of the passage (after 7 d of growth), no difference in $P_{Rv0560c}$ activity was observed between the three types of LacZ. These results show that the OFF kinetics of activity in the passage initially differ slightly between the three types of LacZ. Overall, when linked to tagged LacZs $P_{Rv0560c}$ activity does not return to basal level more quickly than the normal LacZ by the end of the passage. This suggests that $P_{Rv0560c}$ activity is not due to accumulation of LacZ but rather due to continual production of LacZ. Therefore, $P_{Rv0560c}$ activity can only be switched off by dilution of intracellular inducer through growth of the bacteria in multiple passages.

5.2.10 Activity of $P_{Rv0560c}$ in iron-depleted *M. tuberculosis*

Salicylate accumulates in iron-depleted mycobacteria (Ratledge and Winder, 1962). Therefore it was tested if $P_{Rv0560c}$ acivity increased in *M. tuberculosis* grown under iron-limiting conditons. Previous work has shown that activity of low iron-inducible promoters increases in *M. tuberculosis* which have been subjected to 2-3 passages in low iron minimal medium (MMT), indicating that a true state of iron depletion is achieved at this stage (Schreuder and Parish, unpublished).

Activity of $P_{Rv0560c}$ was monitored during several passages in MMT and remained at basal level during the first two passages (Fig 5.16). However, $P_{Rv0560c}$ activity was induced 3-fold (620 MU) during the third passage.

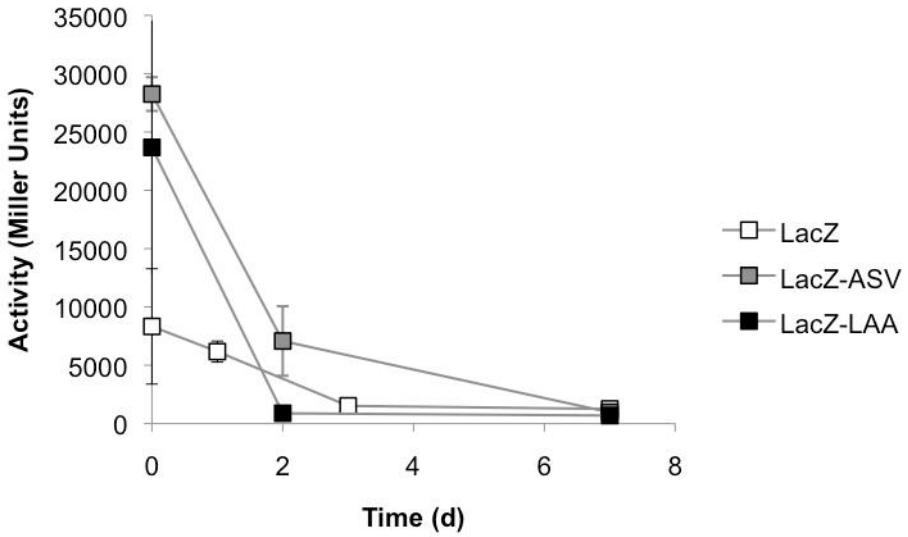

Figure 5.15. Activity of induced $P_{Rv0560c}$ linked to different LacZs during the first passage in salicylate-free medium.

Liquid cultures of *M. tuberculosis* transformants harbouring the $P_{Rv0560c}$ plasmid were grown to mid- (normal LacZ) or late (tagged LacZs) exponential phase in roller bottles with 0.4 mM salicylate. The cells were washed and resuspended in inducer free medium. These cultures were diluted 1/10 and cell-free extracts were prepared between 1 and 7 d after the passage was started. ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU.

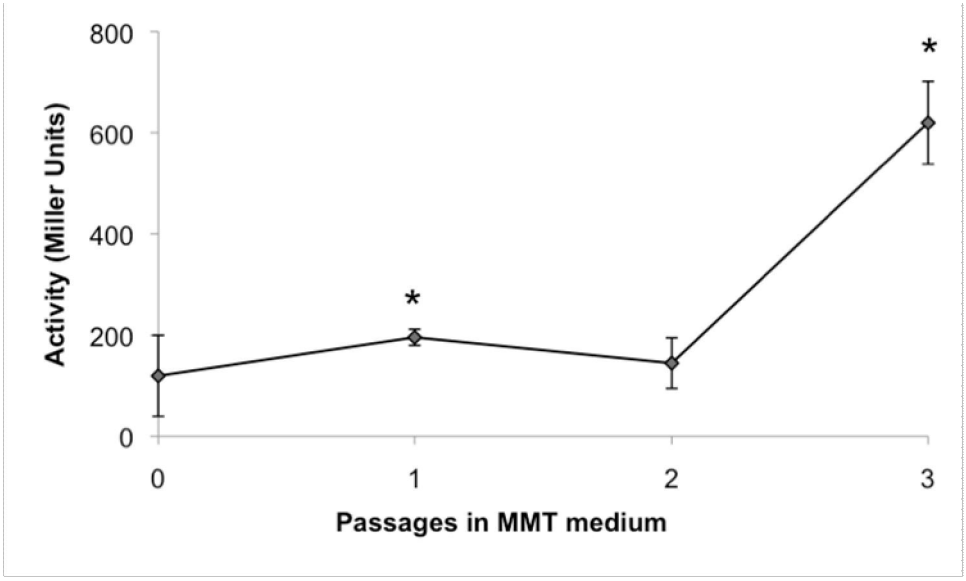

Figure 5.16. $P_{Rv0560c}$ activity under iron-depleted conditions in *M. tuberculosis*.

Liquid cultures of transformants carrying the $P_{Rv0560c}$ plasmid were washed and resuspended in minimal medium (MMT). From this culture, 1 mL was used to inoculate the first passage in MMT medium and cells were grown until mid- to late exponential phase before a second passage was set up. Cells were passaged 3x to ensure complete depletion of any intracellular iron left over from the initial inoculum. Cell-free extracts were prepared from each passage and ß-galactosidase activity in the extracts was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the untreated control is marked by an * ($p < 0.05$).

Thus, the promoter is naturally induced when intracellular levels of iron are believed to be depleted.

5.2.11 Identification of the promoter elements of $P_{Rv0560c}$

Since $P_{Rv0560c}$ is a promising candidate for use in an inducible promoter system, it would be beneficial to identify the promoter elements of $P_{Rv0560c}$ to characterise it further. Three putative -10 elements, two hexamers (P10.1: TGTGTT, P10.2: TATATC) and one heptamer (P10.3: TATATAT) were found immediately upstream of a potential translational start site of Rv0560c (Fig 5.17). To establish which one of the -10 elements was part of the Rv0560c promoter, the first and last T residues were mutated to a G using SDM.

Mutation of P10.1 slightly increased the basal activity but abolished induction by salicylate (Table 5.1). This indicates that this hexamer is not part of the promoter but more likely to be part of the regulatory region. Mutation of P10.2 completely deregulated the promoter, indicating that this might be a site where a regulatory protein binds. As this mutation caused the promoter to be constitutively active at

maximal level one would predict that the regulator is a repressor. Mutation of P10.3 abolished promoter activity to a level seen in the empty vector control. This clearly shows that this sequence is part of the -10 element of the Rv0560c promoter. Closer inspection of the sequence shows that this might be an extended promoter due to the presence of a TGN motif (in this case TGA) directly in front of the -10 element.

5.2.12 Promoter engineering

$P_{Rv0560c}$ could be a promising inducible promoter for use in a regulated expression system. It shows dose-responsive behaviour to its inducer, and strong and stable induction which would make it ideal for use in an antisense setting.

However, it still has a basal level of activity in the absence of inducer, which would impair tight control of expression in a direct promoter replacement setting. Since the -10 element of $P_{Rv0560c}$ was identified, promoter engineering to reduce basal activity, yet retain inducibility, was attempted. SDM was carried out on each residue of the extended -10 element (TGATATATAT).

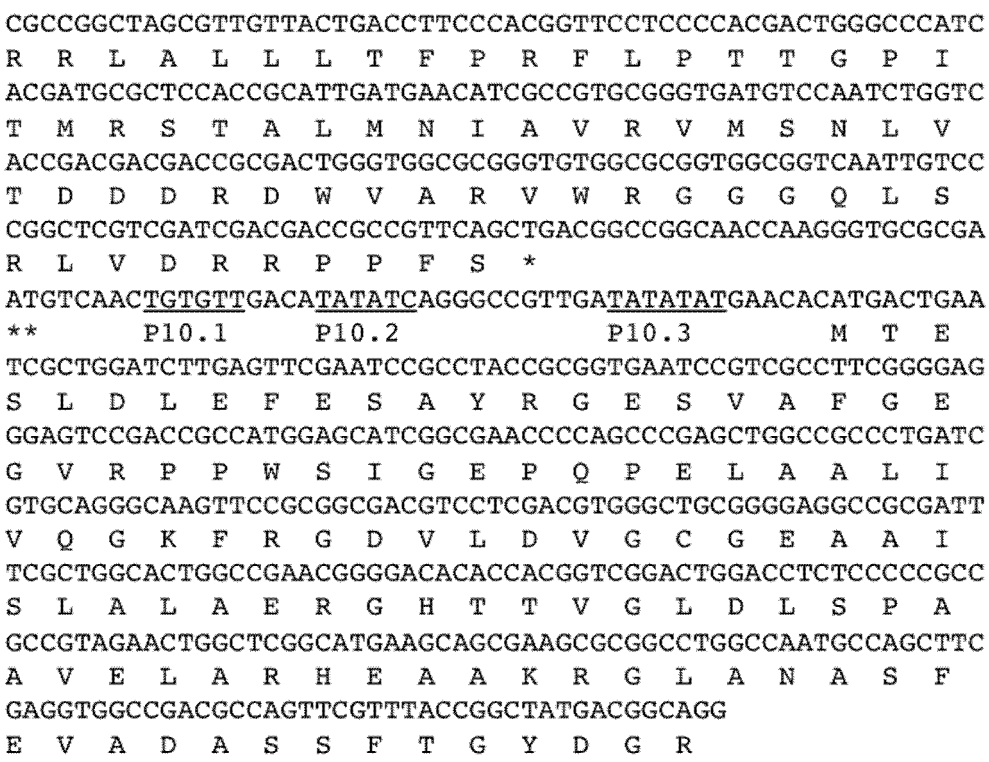

Figure 5.17. DNA sequence in upstream region $P_{Rv0560c}$.

Protein sequences of Rv0561c and Rv0560c are shown below the DNA sequence. Potential -10 promoter elements (P10.1, P10.2 and P10.3) are underlined. The predicted -35 element and the confirmed extended -10 element are shaded in grey. The predicted translation start site of Rv0560c according to TubercuList is marked with **.

Plasmid	SDM	Sequence	Activity (MU) uninduced	Activity (MU) induced	Induction ratio
pDS167	n.a.	wild type	192 ± 41	8482 ± 391	44
pDS180	P10.1	**G**GTGT**G**	261 ± 10*	293 ± 96*	1
pDS181	P10.2	**G**ATA**G**C	15521 ± 1205*	16961 ± 96*	1
pDS182	P10.3	**G**ATATA**G**	10 ± 2*	11 ± 4*	1

Table 5.1. Effect of SDM on putative -10 elements in *M. tuberculosis*.

Three putative -10 elements located upstream of the potential translational start site of Rv0560c were subjected to site directed mutagenesis. Residues highlighted in bold were mutated from T to G. 10 mL aliquots from rolling mid to late exponential phase cultures were subjected to treatment with 0.4 mM salicylate for 4 d. Cell-free extracts were prepared from the cultures before (uninduced) and after (induced) the treatmen. ß-galactosidase activity of the samples was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the unmutated wild type is marked by an * ($p < 0.05$).

Mutation of the first two residues of the extended promoter motif (TGA) had an interesting effect (Table 5.2). Basal activity was increased but salicylate repressed the promoter 2-fold. Mutation of the first residue alone caused $P_{Rv0560c}$ to be constitutively active, but at a higher level than the wild type basal activity. Whereas mutation of the second residue resulted in a promoter of the same strength, but showing only a 2-fold induction. The diverse effects of these mutations were unexpected since they affect promoter regulation as well as strength.

Mutations carried out on the heptamer (TATATAT) affected promoter strength as well as fold induction. Mutation of the second base in the motif reduced basal activity to a very low level but retaining some inducibility (7-fold). Mutation of the third residue had no effect on basal activity, but inducibility in response to salicylate was weakened. Mutations of the last three residues created promoters with reduced basal activity but with strong induction.

These results show that promoter engineering can be successfully used to create a mutant promoter with a lower basal level of activity but good fold induction. Such a promoter would be suitable for use in a direct promoter replacement scenario, since there is little or low activity in the absence of inducer.

5.2.13 Effect of SDM on the operator region

Mutation of P10.2 caused $P_{Rv0560c}$ to be constitutively active at maximal level, suggesting a loss of repression through mutation of a putative binding site for a negative regulator. The mutation is situated immediately downstream of a putative -35 element (TTGACA). Mutating the operator region (between the -35 and -10 elements) might help define a binding motif for the $P_{Rv0560c}$ regulator. To find out if and which other residues between the -35 and -10 elements could be involved in regulation, each two residues were targeted by SDM in turn. The operator mutations can be classified broadly into two categories. First, mutations of the first and third pair of bases (O1 and O3) increased the basal level of activity of $P_{Rv0560c}$, but the promoter could still be induced (over 10000 MU) (Table 5.3). This suggests that binding of the regulator might be slightly reduced by these mutations and repression of promoter activity would be less tight. Secondly, mutations

Plasmid	Sequence	Activity (MU) uninduced	Activity (MU) induced	Induction ratio
pDS167	TGA TATATAT	192 ± 49	8482 ± 391	44
pDS183	**CC**A TATATAT	816 ± 70*	382 ± 235*	0.5
pDS184	**C**GA TATATAT	2037 ± 588*	2260 ± 427*	1
pDS185	T**C**A TATATAT	192 ± 41	414 ± 214*	1
pDS186	TGA T**G**TATAT	19 ± 3*	133 ± 30*	7
pDS187	TGA TA**G**ATAT	234 ± 150	3772 ± 221*	16
pDS188	TGA TAT**G**TAT	54 ± 10*	1239 ± 758*	22
pDS189	TGA TATA**G**AT	63 ± 19*	1514 ± 453*	24
pDS190	TGA TATAT**G**T	56 ± 7*	1147 ± 136*	20

Table 5.2. Effect of SDM on the extended -10 element in *M. tuberculosis*.

Residues in the extended -10 promoter motif of Rv0560c were subjected to site directed mutagenesis. Residues highlighted in bold have been mutated from a T to a C or G or from a G to a C. Activity was measured in exponential phase cultures (uninduced) and after exposure to 0.4 mM salicylate for 4 d (induced). Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the unmutated wild type is marked by an * ($p < 0.05$).

Plasmid	SDM	Sequence	Activity (MU) uninduced	Activity (MU) induced	Induction ratio
pDS167	-	TATATCAGGGCCGT	185 ± 7	8031 ± 671	43
pDS211	O1	**GG**TATCAGGGCCGT	1231 ± 132*	12509 ± 3605*	10
pDS212	O3	TATA**GG**AGGGCCGT	5959 ± 680*	17531 ± 8823	3
pDS213	O4	TATATC**GC**GGCCGT	63 ± 21*	3855 ± 2202*	61
pDS217	O5	TATATCAG**CC**CCGT	127 ± 41	3738 ± 740*	29
pDS214	O6	TATATCAGGG**GG**GT	120 ± 3*	3163 ± 53*	26

Table 5.3. Effect of SDM on the $P_{Rv0560c}$ operator region in *M. tuberculosis*.

Residues in the operator of Rv0560c were subjected to site directed mutagenesis. Residues highlighted in bold have been mutated from T or C to G or from G to C. Activity was measured in exponential phase cultures (uninduced) and after exposure to 0.4 mM salicylate for 4 d (induced). Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from pSM128 (empty vector) was 8 ± 3 MU. A significant difference compared to the unmutated wild type is marked by an * ($p < 0.05$).

of the remaining residue pairs (O4, O5 and O6) reduced the activity of $P_{Rv0560c}$ under uninduced conditions and limited induction of the promoter. Whilst the second group of mutations has interesting effects, the fact that neither of them cause constitutive activation of $P_{Rv0560c}$ indicates that these residues are not involved in binding of a repressor, although they do play a role in promoter strength.

5.3 Discussion

The aim of this project was to develop alternatives to the existing inducible promoter systems available for use in *M. tuberculosis*. A salicylate-responsive gene was selected from microarray and protein studies, its promoter identified and tested for inducibility in response to salicylate by linking it to promoterless reporter genes. The upstream region of Rv0560c, a benzoquinone methyl transferase, was shown to contain a promoter highly inducible in response to salicylate treatment ($P_{Rv0560c}$). In *M. tuberculosis,* $P_{Rv0560c}$ is a dose-responsive promoter that can be strongly and stably induced to a high level of activity (up to 100-fold induction) by treatment with 0.4 mM salicylate. Moreover, salicylate does not kill *M. tuberculosis* when used at this concentration. Lowering the concentration of inducer can be used as an additional level of control of $P_{Rv0560c}$ activity. The slow induction kinetics and high activity of $P_{Rv0560c}$ observed here were highly reproducible even though slight variations in the basal level and the fold induction seen after three days were observed (data not shown). $P_{Rv0560c}$ can be reliably induced to a very high level of activity, making it ideal for overexpression of a gene of interest. Furthermore, it is a very promising candidate for use in an inducible promoter system. $P_{Rv0560c}$ can also be induced to varying degrees by structural analogues of salicylate such as aspirin and PAS, but not by benzoate. It is interesting to note that in a study by Sun and colleagues (Sun *et al.*, 2001), aspirin (of which salicylate is a more stable breakdown product) did not induce Rv0560c expression in the H37Ra strain. This may be explained by differences in the half-life of aspirin or differences in the regulator of $P_{Rv0560c}$ between these two strains. Compounds that can interfere with isoprenoid quinone action and are structurally related to salicylate (such as fenofibrate or gemfibrozil) also induce $P_{Rv0560c}$ activity. Induction kinetics of another inducer, gemfibrozil, are the same as has been observed with salicylate. Thus the use of an

alternative inducer also provides an additional level of control over $P_{Rv0560c}$ activity.

The stable induction kinetics of $P_{Rv0560c}$ seem to be its weakness, since $P_{Rv0560c}$ activity cannot be switched off by removal of salicylate from the growth medium. The failure to switch off $P_{Rv0560c}$ in this way must be due to continued activity as a result of residual levels of intracellular salicylate or a locking of the regulator in a form that causes continued promoter activation. However, the former hypothesis seems to be more likely to be true. $P_{Rv0560c}$ activity can only be brought down to basal level by cell division and growth of the bacteria. This may be due to dilution of intracellular levels of accumulated salicylate. Salicylate content of bacteria during $P_{Rv0560c}$ induction could be monitored to verify this. Attempts to employ the non-inducer benzoate to switch off $P_{Rv0560c}$ activity were unsuccessful, as OFF kinetics were not different in the absence or presence of benzoate. This may be due to benzoate being unable to displace salicylate at the concentration tested or its affinity for binding to the regulator may be less than that of salicylate.

Cloning an upstream region in front of a promoterless reporter gene is a fast way of identifying the presence of a promoter in this sequence. In order to define the promoter elements of $P_{Rv0560c}$, the effect of SDM on three putative -10 elements was characterised in *M. tuberculosis*. $P_{Rv0560c}$ was found to be an extended promoter with a perfect match to the *E.coli* -35 hexamer consensus. $P_{Rv0560c}$ is a strong promoter that might be suitable for overexpression of a gene of interest, but the relatively high basal activity in the absence of inducer would be a problem for direct promoter replacement studies. An attempt was made to reduce the $P_{Rv0560c}$ basal activity whilst retaining inducibility by promoter engineering. Each residue of the -10 element was mutated by SDM and the effect on promoter activity under uninduced and induced conditions was assessed in *M. tuberculosis*. Mutations that lowered the basal level of activity by half or more, and where inducibility was retained, were indeed obtained. The mutated $P_{Rv0560c}$ would be more suitable for use in direct promoter replacement studies to minimise expression of a gene of interest in the absence of an inducing stimulus.

The Rv0560c promoter appears to be regulated by a repressor which binds to residues directly downstream of the -35 element. This view is supported

by the fact that, when placed on a multicopy plasmid, $P_{Rv0560c}$ is constitutively active at maximal level, possibly due to dilution of the regulator (data not shown). Mutations in the operator region identified residues TATATC to be involved in repressor binding. In terms of the regulation of $P_{Rv0560c}$, it is interesting to note that the promoter was also highly inducible by salicylate in *M. smegmatis*. Furthermore, mutations on the -10 element and residues in the operator region had a similar effect to what was observed in *M. tuberculosis* (data not shown). In addition to this, $P_{Rv0560c}$ is also salicylate-inducible in the model organism *M. marinum* (Ahmed, Schuessler and Parish, unpublished). This suggests that the regulator of the Rv0560c promoter is conserved between these three mycobacteria.

The precise function of Rv0560c is unknown, even though it has been implicated in menaquinone biosynthesis (Garbe, 2004; Sun *et al.*, 2001). Rv0560c is not regulated by IdeR, but $P_{Rv0560c}$ activity was induced to a low level under conditions of iron depletion when intracellular levels of salicylate are naturally elevated. This indicates that Rv0560c is upregulated and may play an important role under these conditions. Biological relevance of the fact that $P_{Rv0560c}$ can be induced to an activity much greater than observed under naturally occuring conditions by addition of salicylate to growth medium, and its slow induction kinetics are intriguing. It would be interesting to elucidate the function of Rv0560c and salicylate by characterising gene and protein expression in a Rv0560c mutant grown under conditions of low iron as well as in the presence of salicylate.

In conclusion, the data presented here show $P_{Rv0560c}$ to be an endogenous inducible promoter which could be used for an inducible promoter system for application in *M. tuberculosis*.

6. Application of P_{27} and $P_{Rv0560c}$ as inducible promoter systems

6.1 Introduction

In mycobacteria, inducible promoters have been used to regulate expression of a gene of interest either by direct promoter replacement (Carroll *et al.*, 2005; Chalut *et al.*, 2006; Ehrt *et al.*, 2005; Gandotra *et al.*, 2007; Guo *et al.*, 2007) or by antisense knockdown (Blokpoel *et al.*, 2005; Forti *et al.*, 2009; Kaur *et al.*, 2009; Parish and Stoker, 1997; Rao *et al.*, 2008).

Regulated promoters have been used successfully in an antisense setting to knockdown expression of target genes such as *hisD*, *ftsZ*, *rpoB, dosR* or *narK2* in both fast- and slow-growing mycobacteria (Blokpoel *et al.*, 2005; Kaur *et al.*, 2009; Parish and Stoker, 1997; Rao *et al.*, 2008). In this scenario, conditional silencing of a gene of interest is regulated by expression of antisense RNA through an inducible promoter system. This can be achieved by linking an inducible promoter system to the full length of a gene in an antisense direction. Translation of the mRNA of the gene will be prevented as pairing of the sense and antisense RNA strands blocks access of ribosomes to sense RNA (i.e. the mRNA) and also triggers degradation of double standed RNAs through intracellular RNAses.

One of the objectives of this study was to assay the most promising inducible promoters using reporter genes to determine whether differential expression really occurs and to test these using mycobacterial genes by generating knockdown strains using both antisense and sense approaches.

The strong promoters P_{26}, P_{27} and P_{hspx} were all found to be growth-dependent and already active at a high level in early exponential phase. This rules-out their use for direct promoter replacement since their promoter activity is not tightly regulated. However, the strength of the promoters and the fact that they reach maximal induction during stationary phase could make them suitable for use in an antisense setting to knockdown a gene of interest during stationary phase. P_{27} was the strongest of the hypoxia-responsive promoters and might be suitable for use in an antisense setting.

The wild type $P_{Rv0560c}$ has a weak to medium basal activity but can be stably induced to more than 100-fold in response to salicylate. The high maximal activity and stable induction of $P_{Rv0560c}$ make it an ideal promoter to use in an antisense setting. Antisense knockdown of a gene of interest could be induced by growing bacteria harbouring a knockdown plasmid in the presence of salicylate. In addition to this, mutant $P_{Rv0560c}$ with a reduced basal level could be used to create a conditional mutant through direct promoter replacement.

6.2 Results

6.2.1 Testing P_{27} and $P_{Rv0560c}$ in an antisense setting using *lacZ*

In order to test if P_{27} and wild type $P_{Rv0560c}$ could be successfully employed for knockdown of a reporter gene, antisense plasmids were created. LacZ antisense plasmids, controlling expression of full length antisense *lacZ* from P_{27} or $P_{Rv0560c}$, were constructed and transformed into TBG117, a strain of *M. tuberculosis* which contains the constitutively active promoter $P_{Rv0561c}$ linked to *lacZ* integrated into the mycobacterial genome. If successful knockdown of *lacZ* through inducible expression of LacZ antisense RNA occurred, ß-galactosidase activity (due to *lacZ* expression from $P_{Rv0561c}$) would be reduced under inducing conditions (Fig 6.1).

For $P_{27,}$ ß-galactosidase activity in *M. tuberculosis* transformed with either an empty vector control or the P_{27}-LacZ knockdown plasmids was measured during exponential phase (non-inducing conditions) and stationary phase (inducing conditions) growth.

During exponential phase growth, ß-galactosidase activity in the strain carrying the empty vector was 300 MU (Fig 6.2). Promoter activity was not reduced in strains carrying the antisense plasmid. Thus there was no knockdown of *lacZ*. This is as expected since P_{27} is not active at its maximal activity during exponential phase so if antisense RNA was being expressed from P_{27} there may not have been enough of it to cause a marked effect. During stationary phase growth, ß-galactosidase activity was not reduced in strains transformed with the antisense plasmid. Thus there was no reduction in the amount of LacZ present in the cells. This shows that,

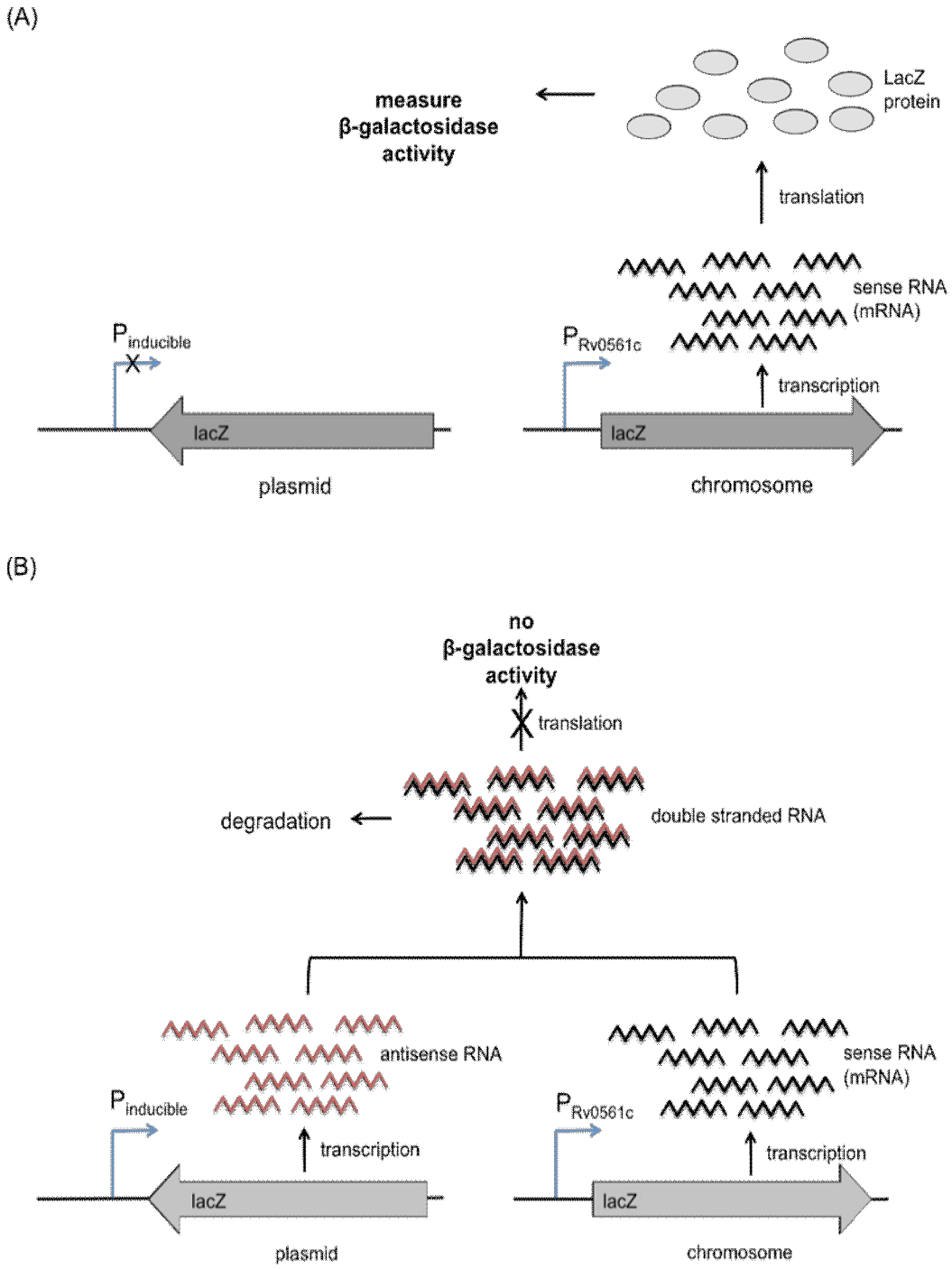

Figure 6.1. **Schematic depiction of inducible gene silencing.**

(A) Under non-inducing conditions, no antisense RNA is produced. LacZ is produced from the constitutively active $P_{Rv0561c}$, and amount of protein produced can be quantified by measuring ß-galactosidase activity. (B) Under inducing conditions, antisense RNA is produced. Antisense RNA binds to its complementary sense RNA and the double stranded complex is not translated into protein, but degraded. Thus less or no LacZ is being produced.

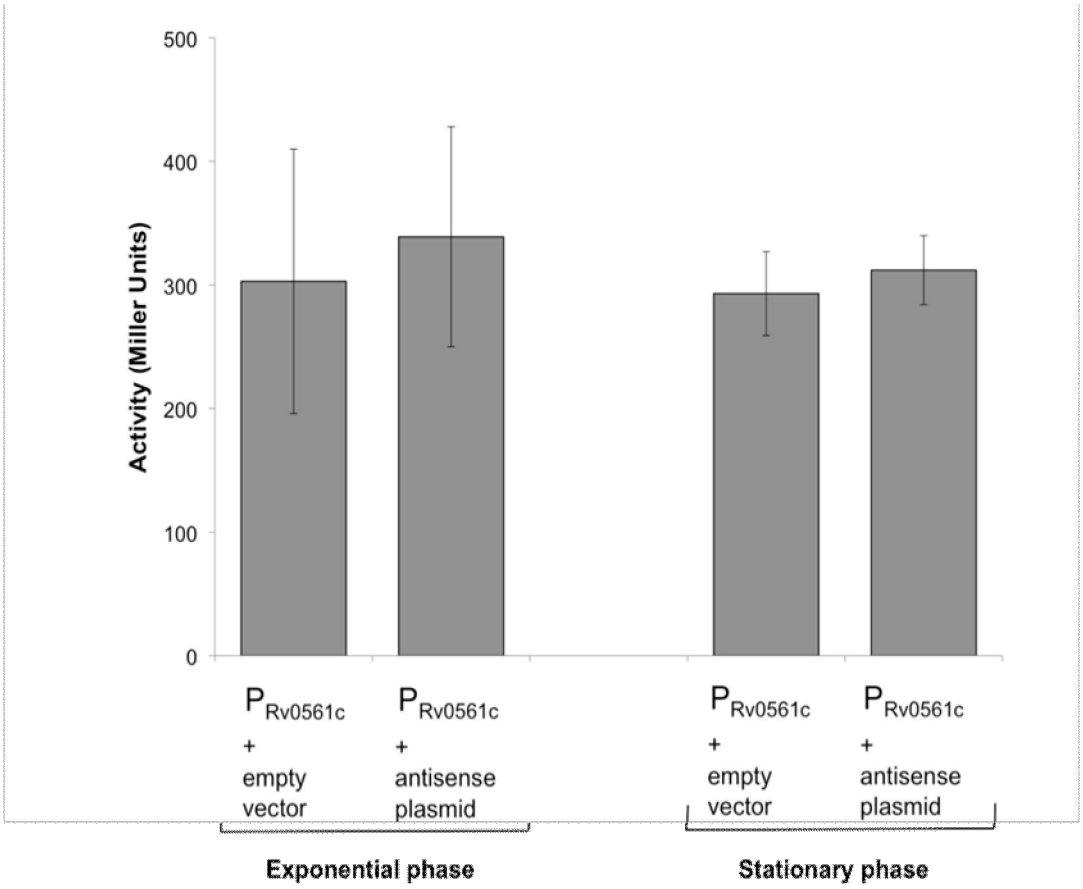

Figure 6.2. Effect of P_{27} antisense LacZ expression on ß-galactosidase activity under non-inducing and inducing conditions in *M. tuberculosis*.

Activities are shown during exponential growth, when P_{27} activity is at its lowest, and stationary phase growth, when P_{27} activity is at its highest, in *M. tuberculosis* and strains co-transformed with the empty vector control and the P_{27} antisense plasmid. Liquid cultures were grown in 7H9 from a starting OD_{580} of 0.1. Samples were taken during exponential (OD_{580} of 0.5) and stationary phase (OD_{580} of 1.5) and promoter activity was measured. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The ß-galactosidase activity from a strain containing only the $P_{Rv0561c}$ was 401 ± 93 MU.

unexpectedly, under conditions where P_{27} is at its most active, no successful knockdown of the reporter gene was achieved.

For $P_{Rv0560c}$, ß-galactosidase activity was measured before (non-inducing conditions) and after (inducing conditions) exposure to salicylate.

In the absence of inducer, ß-galactosidase activity was the same (~300MU) in strains transformed with either the empty vector control or the $P_{Rv0560c}$-LacZ antisense plasmid (Fig 6.3). Thus there was no decrease in amount of LacZ in the absence of inducer in the strain carrying the antisense plasmid. This is was not surprising since $P_{Rv0560c}$ is active at basal level in the absence of inducer, and if some antisense RNA was being expressed from $P_{Rv0560c}$ there may not have been enough of it to cause a marked effect. In the presence of salicylate, ß-galactosidase activity was not reduced in strains carrying the antisense plasmid (Fig 6.3). Thus, surprisingly, there was no knockdown of *lacZ*, even when $P_{Rv0560c}$ was induced to its maximal level.

6.2.2 Testing P_{27} and $P_{Rv0560c}$ in an antisense setting using *rpoB*

An attempt to use P_{27} and $P_{Rv0560c}$ to decrease expression of a reporter protein was unsuccessful. To test if the promoters were able to silence expression of a real mycobacterial gene, antisense plasmids were created choosing *rpoB* as target gene. *RpoB* encodes the ß-chain subunit of RNA polymerase and is essential for growth as well as rifampicin resistance (Miller *et al.*, 1994; Sassetti *et al*., 2003). Antisense plasmids were made by placing full length *rpoB* in an antisense direction downstream of P_{27} or $P_{Rv0560c}$.

For the P_{27}-RpoB antisense plasmid, growth of transformants was assayed in the presence of a range of concentrations of rifampicin to check if there was a reduction in growth or increased sensitivity compared to the vector control during aerobic growth.

Wild type *M. tuberculosis* grew quickly to an OD_{580} of 2 in the absence of rifampicin and when grown with 0.001µg mL^{-1} rifampicin (Fig 6.4A). There was no growth in the presence of ≥0.005 µg mL^{-1} rifampicin until the emergence of resistant clones after 20 d. Resistance of these clones was

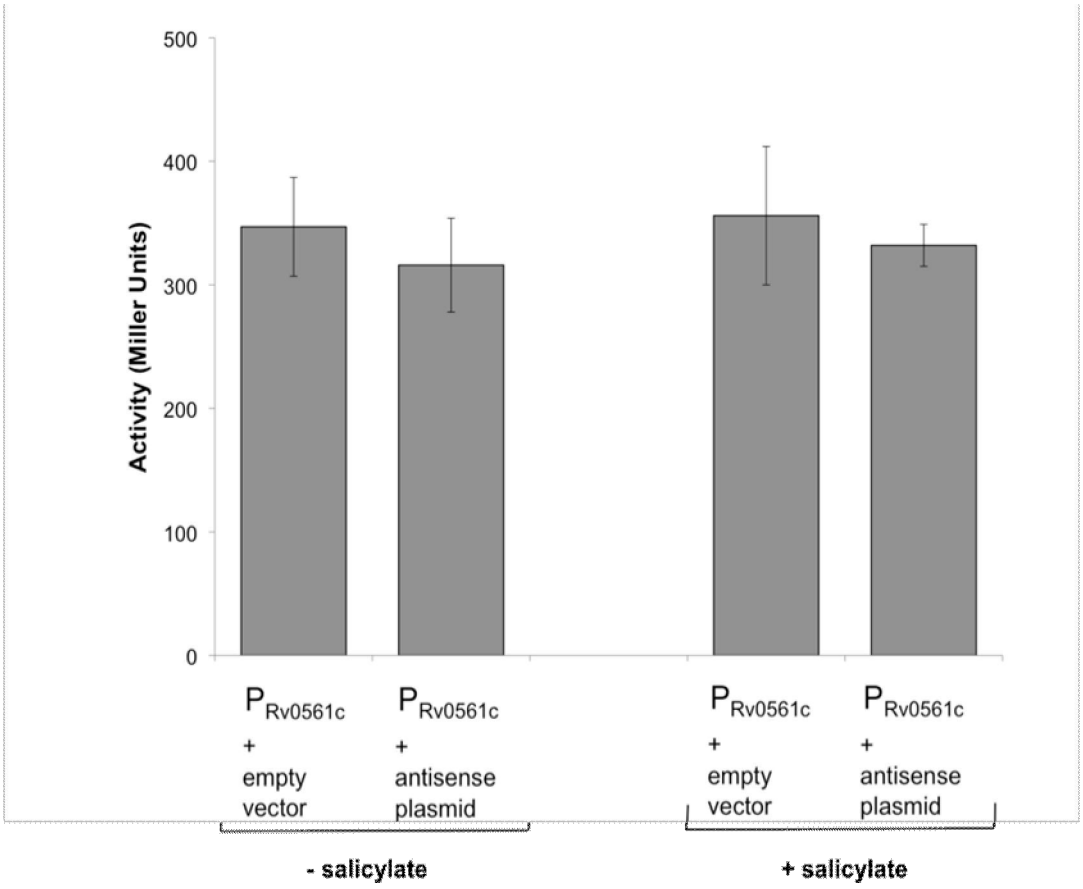

Figure 6.3. Effect of $P_{Rv0560c}$ antisense LacZ expression on ß-galactosidase activity under non-inducing and inducing conditions in *M. tuberculosis*.

Activities are shown in the absence of salicylate when $P_{Rv0560c}$ activity is at its lowest and after $P_{Rv0560c}$ induction through salicylate treatment when $P_{Rv0560c}$ activity is at its maximum. 10 mL liquid cultures of the transformants harbouring the $P_{Rv0561c}$ plasmid and either an empty vector control or the $P_{Rv0560c}$-LacZ antisense plasmid were grown to mid-late exponential phase. Cell-free extracts were prepared before and after exposure to 0.4 mM salicylate for 7 d. Results are the average activity of three independent transformants assayed in duplicate ± standard deviation. Miller Units are amount of O-nitrophenol produced (nmol) per min per mg of total protein. The background activity from a strain containing only the $P_{Rv0561c}$ plasmid was 405 ± 91 MU.

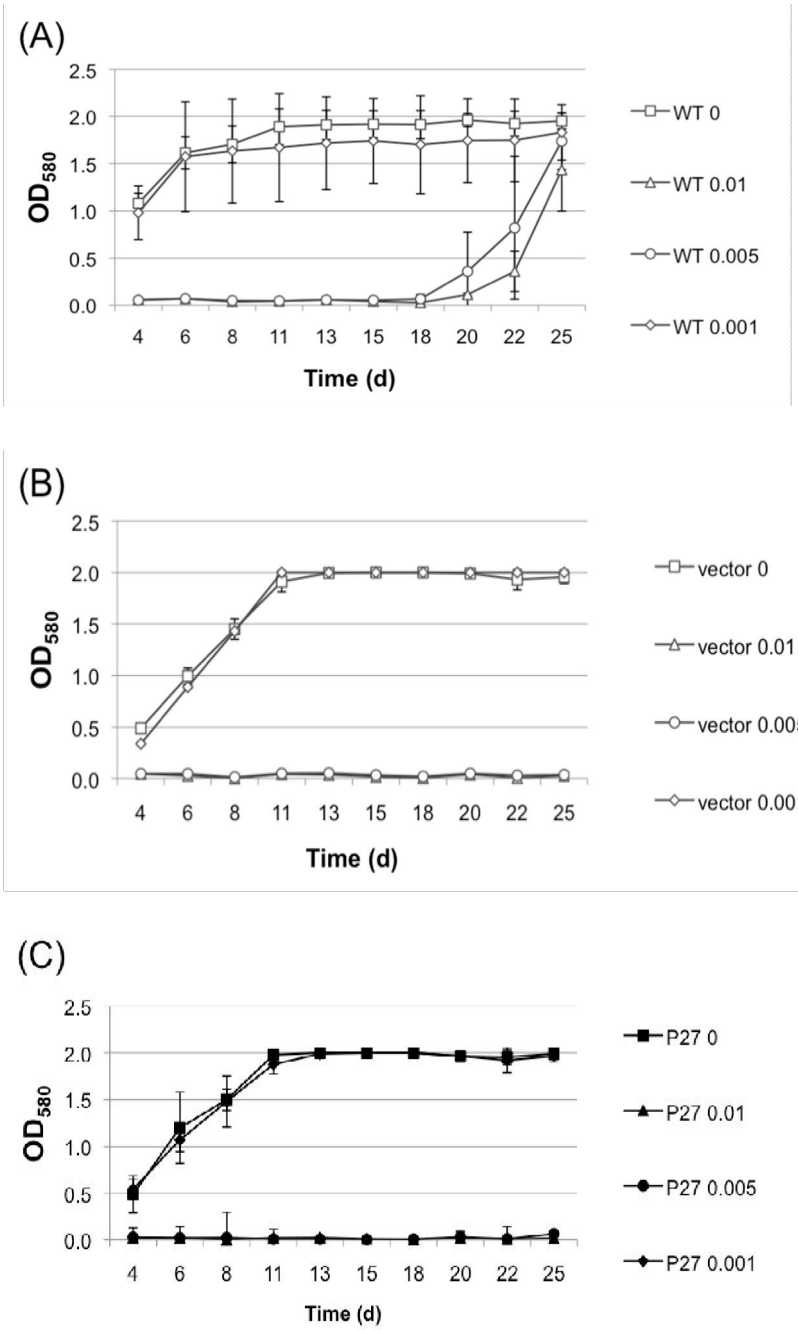

Figure 6.4. **Effect of the P_{27}-RpoB antisense plasmid on growth of *M. tuberculosis* in the presence of rifampicin.**

Growth of (A) *M. tuberculosis* wild type (WT), (B) transformants carrying the empty vector control and (C) transformants carrying the P_{27}-RpoB antisense plasmid ("P27"). Either 0, 0.1, 0.005 or 0.001 μg mL^{-1} rifampicin was added to the growth medium. Liquid cultures of transformants were grown from a starting OD_{580} of 0.02 up to an OD_{580} of 2. The mean OD_{580} of three independently assayed transformants is shown ± standard deviation in relation to time in days.

confirmed by assaying growth of samples of the liquid cultures on solid medium containing 0.02 µg mL^{-1} rifampicin.

M. tuberculosis transformed with the empty vector control followed a similar pattern (Fig 6.4B). In the absence of rifampicin, or in the presence of 0.001 µg mL^{-1} rifampicin, transformants grew slower than the wild type, reaching an OD_{580} of 2 only after 11 d of growth. There was no growth in the presence of the higher concentrations of rifampicin. The vector itself slightly impaired growth of the bacteria and their ability to grow in the presence of certain concentrations of rifampicin slightly, since the additional selection pressure of its antibiotic resistance would be expected to slow down the growth kinetics of the transformants.

Transformants carrying the *rpoB* antisense plasmid under the control of P_{27} behaved in a similar fashion to the transformants carrying the vector control (Fig 6.4C). Unexpectedly, there was no significant difference in growth between the vector control and transformants carrying the antisense plasmid. Therefore the attempt to employ P_{27} for knockdown of *rpoB* was unsuccessful.

Growth of transformants carrying the $P_{Rv0560c}$-RpoB antisense plasmid was assayed in the presence of a range of concentrations of rifampicin with and without salicylate added to the medium.

In the absence of salicylate, *M. tuberculosis*, transformants carrying the $P_{Rv0560c}$-RpoB antisense plasmid grew as quickly as the empty vector control and displayed the same pattern of rifampicin sensitivity (Fig 6.5A). Thus in the absence of inducer, bacteria did not suffer from any growth defect.

In the presence of inducer, growth of both control and antisense strains tested was slowed down, taking at least 11 d to reach stationary phase (Fig 6.5B). The pattern of rifampicin sensitivity was the same between the vector control and antisense transformants. Thus, surprisingly, under inducing conditions no phenotypic effect i.e. potential knockdown of *rpoB* was observed.

6.3 Discussion

P_{27} was used in an antisense setting to attempt knockdown of *lacZ* or *rpoB* through interaction of antisense and sense RNA. However, when

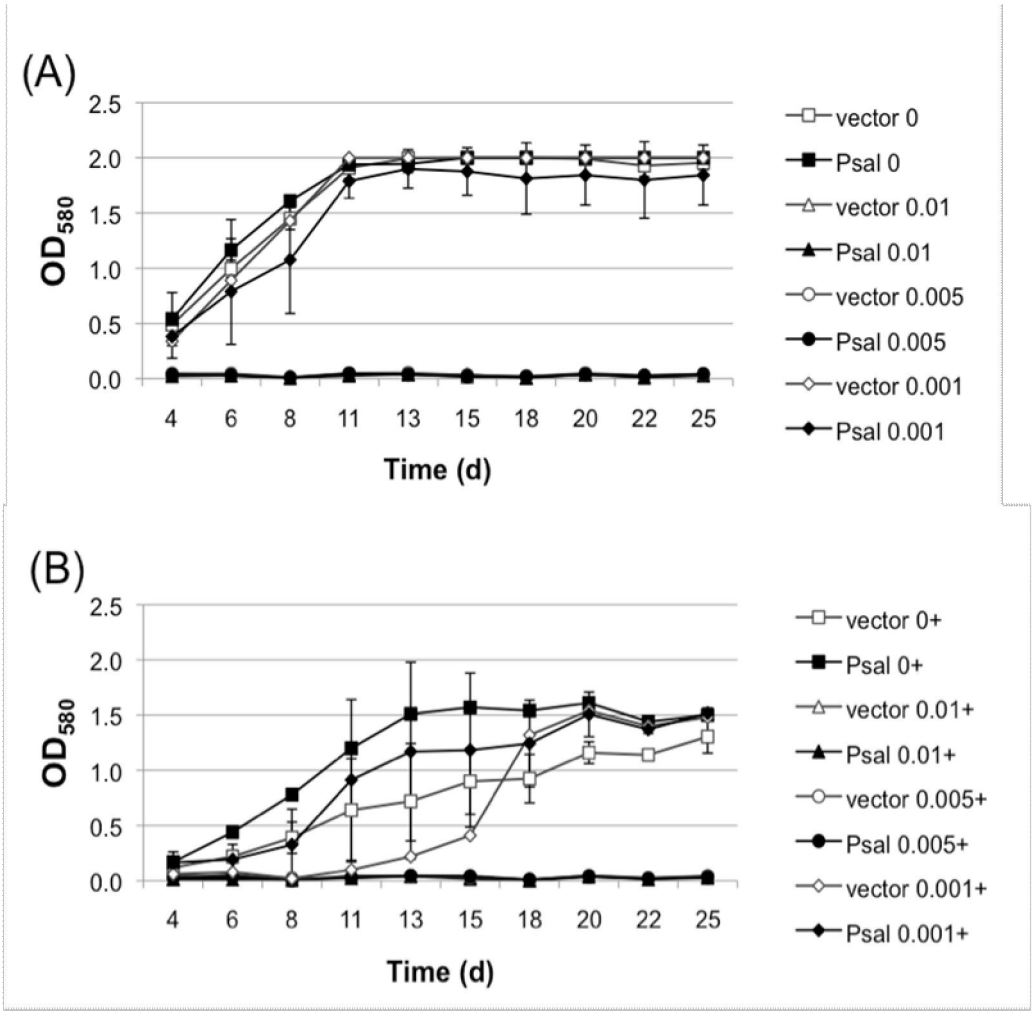

Figure 6.5. Effect of the $P_{Rv0560c}$-RpoB antisense plasmid on growth of *M. tuberculosis* in the presence of rifampicin.

Growth of transformants carrying the empty vector control (vector) or the $P_{Rv0560c}$-RpoB antisense plasmid ("Psal") was monitored over 25 d (A) in the absence of inducer and (B) in the presence of 0.4 mM salicylate (+). Either 0, 0.1, 0.005 or 0.001 µg mL^{-1} rifampicin was added to the growth medium. Liquid cultures of transformants were grown from a starting OD_{580} of 0.02 up to an OD_{580} of 2. The mean OD_{580} of three independently assayed transformants is shown ± standard deviation in relation to time in days.

expression of antisense RNA was induced from P_{27}, no significant difference in either amount of LacZ produced or ability to grow in the presence of rifampicin was observed between strains containing the P_{27} antisense plasmids or the empty vector control. This may have been due to several factors: failure of P_{27} activity to induce expression of mRNA may have been due to the fact that P_{27} was on a multicopy vector in this setting, whereas previously its activity was characterised on a single copy vector; multiple copies of P_{27} being present may have diluted out the regulator DosR so that there was not enough phosphorylated DosR available to activate P_{27} to its full strength. Another possibility is that no antisense mRNA was expressed from P_{27} or that the antisense mRNA failed to bind to its sense mRNA.

The wild type $P_{Rv0560c}$ was used in an antisense setting to attempt knockdown of *lacZ* in a strain of *M. tuberculosis* that constitutively expressed *lacZ* from an integrated plasmid. $P_{Rv0560c}$ was also used to attempt knockdown of the mycobacterial gene *rpoB*. However, both attempts at using $P_{Rv0560c}$ in an antisense setting failed. This may have been due to the fact that in this setting $P_{Rv0560c}$ was on a replicative plasmid, since $P_{Rv0560c}$ activity was found to be deregulated on a multicopy plasmid and be constitutively active (data not shown). Mutations may have accumulated in the antisense plasmids, rendering $P_{Rv0560c}$ less active or inactive, and as a result no or little antisense RNA would have been expressed. To confirm this possibility, one would have to extract the antisense plasmids from the strains and check the plasmids for mutations in the promoter region by sequencing. Alternatively, one could assay if and how much antisense RNA was expressed in each of the strains under non-inducing and inducing conditions. Other explanations for lack of a knockdown phenotype may have been that the sense and antisense RNAs failed to pair up due to effect of secondary structures or unspecific binding. Another possibility may be that there was insufficient knockdown occuring to evoke a phenotypic effect.

In conclusion, both P_{27} and $P_{Rv0560c}$ looked to be good candidates for use as inducible promoters in an antisense setting, but attempts to prove their utility were unsuccessful. This may have been due to failure of the antisense RNA to be transcribed or failure of this RNA to bind to and cause degradation of its cognate sense RNA or there being insufficient knockdown to observe an effect on growth. There are several studies that have successfully used an inducible promoter in an antisense setting to silence expression of a target

gene in mycobacteria, although why in some cases this approach fails to or does succeed is poorly understood (Blokpoel *et al.*, 2005; Forti *et al.*, 2009; Kaur *et al.*, 2009; Parish and Stoker, 1997; Rao *et al.*, 2008).

7. General discussion

Currently, there are several inducible promoter systems available for use in *M. tuberculosis* (Blokpoel *et al.*, 2005; Carroll *et al.*, 2005; Ehrt *et al.*, 2005; Forti *et al.*, 2009; Gandotra *et al.*, 2007; Kaur *et al.*, 2009; Lee *et al.*, 2008a; Pandey *et al.*, 2009). The ideal regulated expression system should be tightly switched off in the absence of inducer, quickly respond to non-toxic concentrations of inducer, allow regulation over a range of expression levels and if used for the study of pathogenesis, permit gene regulation during an *in vivo* infection. None of the promoter systems currently available for use in mycobacteria fulfill all of these properties. (Blokpoel *et al.*, 2005; Carroll *et al.*, 2005; Ehrt *et al.*, 2005; Ehrt and Schnappinger, 2006; Forti *et al.*, 2009; Gandotra *et al.*, 2007; Kaur *et al.*, 2009; Lee *et al.*, 2008a; Pandey *et al.*, 2009).

The aim of this work was to contribute to the discovery of new drug targets by the development of novel inducible promoter systems to be used as alternatives to the existing ones. To ensure that these systems would be functional and genetically stable in *M. tuberculosis*, it was decided to find and use endogenous inducible promoters of *M. tuberculosis* for the purpose of this work.

The literature was mined for *M. tuberculosis* genes that were induced in response to antibiotics (tetracycline and streptomycin), hypoxia and salicylate. The upstream regions of these genes were tested for promoter activity by construction of plasmids using promoterless reporter genes *lacZ* or *gfp*. These plasmids were used to determine whether inducible expression occurred *in vitro*.

Upstream regions of five tetracycline-, one streptomycin- and one ethanol- or SDS-responsive gene were tested for promoter activity and inducibility in *M. tuberculosis*. Most of the upstream regions contained no or only very weak promoters and were not found to be inducible. After re-amplification of three upstream regions, two ($P_{Rv1015c}$ and P_{whiB6}) were found to have weak to medium strength promoter activity, but they were not found to be inducible. Failure to find suitably inducible promoters, may have been due to the apparent induction of the candidate genes being a general as opposed to a

specific effect or the upstream regions tested not containing a functional promoter. Upstream regions of four hypoxia- and NO- responsive genes, which are part of the DosR regulon, were tested for promoter activity and inducibility in *M. tuberculosis*. Even though promoter activity, of varying strength, was found for all four upstream regions (P_{27}, P_{26}, P_{25} and P_{hspx}), only one of the promoters (P_{26}) was found to be inducible by stress stimuli such as ethanol, hypoxia or standing culture conditions. P_{27} was not tested under these conditions. High variations in the results may have masked inducibilty of P_{hspx}. All four promoters were induced during stationary phase growth in *M. tuberculosis* and for all, except one (P_{25}), this behaviour was dependent on the regulator DosR. A second regulator (Rv2625c) was identified for one of this set of stationary phase-inducible promoters (P_{26}). The upstream regions of two genes that could potentially be in an operon were tested for promoter activity and inducibility in response to salicylate. Both regions contained functional promoters and one of them, $P_{Rv0560c}$, was found to be salicylate-inducible.

Out of all the promoters tested, P_{27} and $P_{Rv0560c}$ appeared to be the most promising. They were the promoters with the highest activity upon induction. P_{27} could be induced up to 8-fold during stationary phase and $P_{Rv0560c}$ could be induced up to 100-fold after treatment with salicylate. They were tested in an antisense approach to attempt knockdown of the reporter gene *lacZ* or the mycobacterial gene *rpoB*. However, silencing of *lacZ* did not occur from either of the promoters used and no phenotypic effect was seen when the promoters were used to silence *rpoB*. Further experiments would be needed to investigate why this attempt failed, for example one would have to verify that the promoters actually express the antisense RNA. Furthermore, antisense plasmid design might have to be improved to make the systems work.

$P_{Rv0560c}$ has the most potential for use as an inducible expression system. Unlike P_{27}, its activity is independent of growth and thus it is only induced in response to salicylate. In addition to it responding to a specific inducer, it is also dose-dependent. The magnitude of fold induction (100-fold) and the slow induction kinetics (three days to reach maximal activity) of $P_{Rv0560c}$ are comparable to those of the widely used tetracycline-inducible Tet-ON system (Ehrt *et al.,* 2005) as well as the pristinamycin-inducible system (Forti *et al.*, 2009). Previous problems of background activity with the Tet-

ON system have been improved by codon optimisation of the regulator of the system and the pristinamycin-inducible system did not suffer from this problem. Even though wild type $P_{Rv0560c}$ suffers from background activity in the absence of inducer, the fact that mutations could alleviate this problem should make $P_{Rv0560c}$ just as attractive as the new anhydrotetracycline- or the pristinamycin-inducible system. In addition to this, the salicylate-inducible system should, like the exisiting antbiotic-inducible systems, be functional for use during host infection (Forti *et al.*, 2009; Gandotra *et al.*, 2007).

Selection of genes that are not truly inducible, as seen for most of the antibiotic-responsive genes, should be avoided. One would have to ensure that upregulation of a gene under a certain condition is a specific as opposed to a general effect, before testing potential promoters of the gene for inducibility. Hence, selection of candidate genes could be improved by either carrying out another microarray or (q)RT-PCR study to check that the findings in the literature are reproducible. In addition to this, protein studies such as 2D gel electrophoresis or Western blots could also be employed to ensure that a gene was specifically upregulated by a certain stimulus. This might ensure selection of truly differentially expressed genes, promoters of which would be worth identifying and characterising. In order to avoid testing upstream regions that do not actually contain a functional promoter, it would be advisable to closely inspect if the genes upstream or downstream of the candidate gene were also induced in response to a certain stimulus and if genomic localisation would indicate the gene to be part of an operon with preceeding genes. One could confirm if a gene is part of an operon or not with a Northern blot or RT-PCR. Failing that, one could proceed to test any potential promoter for promoter activity. However, this would be time-consuming and laborious. In conclusion it might be more practical to select genes which have been shown to be truly differentially expressed in more than one study, as has been done with the hypoxia- and salicylate-inducible promoters.

Further work on the hypoxia-inducible promoters could include repeating promoter inductions with ethanol or DETA/NO during exponential phase in order to see if the promoters could be induced beyond their basal level of activity during early stages of growth. In the future, it might be more prudent to exclude any DosR-regulated genes from attempts to find hypxoia-

inducible promoters, as thus one would select promoters whose activity does not increase with growth.

In order to further develop the use of $P_{Rv0560c}$ as an inducible promoter system it would be useful to determine the induction kinetics of the mutant $P_{Rv0560c}$(s), to see if they differ from the wild type promoter. Similarily, the use of the alternative inducers aspirin and PAS could be characterised in more depth in order to find out if they could be used for additional control over $P_{Rv0560c}$ activity. One distinctive feature of the inducible systems that are currently used in *M. tuberculosis* is that the regulator of the inducible promoter is known (Ehrt *et al.*, 2005; Forti *et al.*, 2009; Kaur *et al.*, 2009; Lee *et al.*, 2008a; Pandey *et al.*, 2009). Having detailed knowledge of the regulator of an inducible promoter would be advantageous, as it allows additional levels of control over the amount of regulator present in a cell as well as the regulator's response to the inducing stimulus. The regulator of $P_{Rv0560c}$ could be identified by DNA binding studies or creation of a transposon mutant library. Since it is likely that $P_{Rv0560c}$ is regulated by a conserved mechanism in mycobacteria, attempts to find the regulator of $P_{Rv0560c}$ could be carried out in the model organism *M. smegmatis*. Once the regulator has been identified, further work could be carried out to try to isolate a mutant of the regulator that would allow the promoter to be switched off in response to salicylate treatment -similarly to the Tet-OFF system.

To conclude, the collective data generated during this project has shown that there are endogenous promoters from *M. tuberculosis* which would be suitable for use as inducible expression systems. These could be used as alternatives to the existing systems or be used in conjunction with them. Although investigations into the use of the stationary phase- or salicylate-inducible promoters for controlling expression of a mycobacterial gene have only been commenced and further in depth study would be required, these two expression systems look to be promising alternatives. Furthermore, owing to the fact that regulation of endogenous inducible promoters is distinct from the currently available systems, it would make them ideal for use in combination with these, if one wished to study varying expression levels of more than one gene. In addition to use for elucidation of gene function, the endogenous systems could potentially be used on their own, or in conjunction with others, to identify genes essential for mycobacterial

survival under defined conditions, i.e. potential drug targets; or be used to determine target vulnerability and target specificity. This would contribute to the discovery of new drug targets and aid the development of novel antimycobacterial agents.

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9. Appendices

Appendix 1. Primers used for amplification of upstream regions of antibiotic-responsive genes in this study.

SmaI restriction sites are underlined.

Primer	Sequence (5' to 3')	Purpose
Rv0277cF	CCCGGGGCCACGCCCGGTGCC	Amplification of upstream region of Rv0277c
Rv0277cR	CCCGGGTCGTCGGCCGCAAGC	Amplification of upstream region of Rv0277c
Rv0608F	GCAGGCCAAATTCTGAAACTCT	Amplification of upstream region of Rv0608
Rv0608R	TGATTTTCGCGATCTGCTT	Amplification of upstream region of Rv0608
Rv0748F	GCGGCGTCGGCGGCAGCGCC	Amplification of upstream region of Rv0748
Rv0748R	GAAGGGCGTCCTCGATCA	Amplification of upstream region of Rv0748
Rv1015cF	CCCGGGAGGCGACTGCACACCTGG	Amplification of upstream region of Rv1015c
Rv1015cR	CCCGGGACGCGCCCTTGCC	Amplification of upstream region of Rv1015c
Rv2478cF	CCCGGGTGACAGCGAGTTCGACG	Amplification of upstream region of Rv2478c
Rv2478cR	CCCGGGTCGGCATCGCCGCGGCCG	Amplification of upstream region of Rv2478c
Rv3898cF	GAAAACGAAGCCGAGCTG	Amplification of upstream region of Rv3898c
Rv3898cR	GGGTAGGTCTACGGGGACAGC	Amplification of upstream region of Rv3898c
whiB6F	CTGCGTTGATCCCTTATTCG	Amplification of upstream region of *whiB6*
whiB6R	GGGATCTTGCGTGCAAAG	Amplification of upstream region of *whiB6*
whiB7F	GGATATTTCAGCGCTTCTCG	Amplification of upstream region of *whiB7*
whiB7R	TCGCTCGTCATACAGCAGAG	Amplification of upstream region of *whiB7*
P1015cF	CGCCGGAATTCGTCGTCGCGGCAAAG	Amplification of new upstream region of Rv1015c
P1015cR	CACGGCGCACGACCAGATGGTC	Amplification of new upstream region of Rv1015c
P2487cF	GCTGCGCGCCGGGGCGAATCAG	Amplification of new upstream region of Rv2487c
P2387cR	CGCCTGCACAAACTGCTCGTG	Amplification of new upstream region of Rv2487c
newB6R	CCCCCCGGGGACAAAGCGTTGCAGGTTG	Amplification of new upstream region of *whiB6*

Appendix 2. Primers used for amplification of upstream regions of hypoxia-responsive genes in this study.

SmaI restriction sites are underlined.

Primer	Sequence (5' to 3')	Purpose
HspxF	GGTGCCCGGGATGATCAACCTCCGCTG	Amplification of upstream region of *hspX*
HspxR	GACCCGGGACCGCGGGTGGCGCTGAAC	Amplification of upstream region of *hspX*
Rv2625cF	GGCCCGGGACAGCATCTAC	Amplification of upstream region of Rv2625c
Rv2625cR	CCCCGGGATCACCAACACGCTCCAG	Amplification of upstream region of Rv2625c
Rv2625sF	CAGTTCGTCAAGGCAATCTGC	Amplification of shorter upstream region of Rv2625c
Rv2626cF	CAACCCGGGTCCGCCAGGACC	Amplification of upstream region of Rv2626c
Rv2626cR	CAACCCGGGTCACACCTGCGTTC	Amplification of upstream region of Rv2626c
Rv2627cF	GCCCCGGGGACCGTCGATGTG	Amplification of upstream region of Rv2627c
Rv2627cR	GCCCGGGGGACTCAGGCGAAAAGC	Amplification of upstream region of Rv2627c

Appendix 3. Primers used for RT-PCR in this study.

Primer	Sequence (5' to 3')	Purpose
sigAF	CAGGACACTACGACCAGCAC	Amplification of 5`end of sigA (300 bp)
sigAR	CTCACGTTCGAGGTCTTCGT	Amplification of 5`end of sigA (300 bp)
B27F	GCTTTTCGCCTGAGTCCACCGG TCTTG	Amplification of 5`end of Rv2627c (306 bp)
B27R	CAAAAAATCCGCTAGGCTTCTCC AG	Amplification of 5`end of Rv2627c (306 bp)
B26F	GAACGCAGGTGTGACCTGTGTT GGC	Amplification of 5`end of Rv2626c (398 bp)
B26R	GGGCCATGGGCGAGCAGATTGC CTTG	Amplification of 5`end of Rv2626c (398 bp)
B25F	GCGGATCGCCGGGTTTGTG	Amplification of 5`end of Rv2625c (287 bp)
B25R	CTTTGGGCGTCTTTGCCTCG	Amplification of 5`end of Rv2625c (287 bp)
J67F	GTGGACGCGCCACGGACACAG	Amplification of the Rv2627c-Rv2626c junction (601 bp)
J67R	GCCAACACAGGTCACACCTGCG TTC	Amplification of the Rv2627c-Rv2626c junction (601 bp)
J56F	CAAGGCAATCTGCTCGCCCATG GCCCTC	Amplification of the Rv2626c-Rv2625c junction (137 bp)
J56R	CACAAACCCGGCGATCCGCCCA AG	Amplification of the Rv2626c-Rv2625c junction (137 bp)

Appendix 4. Primers used for amplification of salicylate-responsive genes in this study.

SmaI restriction sites are underlined.

Primer	Sequence (5' to 3')	Purpose
UR561F	CCCCCCGGGGGATCGCGACGTTGTTAC	Amplification of upstream region of Rv0561c
UR561R	CCCCCCGGGCCGCCAGCCACTTAC	Amplification of upstream region of Rv0561c
UR560F	CCCCCCGGGGCGCCGGCTAGCGTTGTTAC	Amplification of upstream region of Rv0560c
UR560R	CCCCCCGGGCCTGCCGTCATAGCCGGTAAACG	Amplification of upstream region of Rv0560c

Appendix 5. Primers used for SDM in this study.

Primer	Sequence (5' to 3')	Purpose
Rv25mutF	GCACGAAAGGCTTTGCCCG CCAGCATCGGAGG	SDM of putative -10 element from CTTTAT to CTTTGC in upstream region Rv2625c
SDM-1026F	GCTAGTGGGACAGCATCTG CCACGTCGATGCGAACGC	SDM of putative -10 element from TCTACT to TCTGCC in upstream region Rv2625c
SDM-1026F	GCGTTCGCATCGACGTGGC AGATGCTGTCCCACTAGC	SDM of putative -10 element from TCTACT to TCTGCC in upstream region Rv2625c
LAAF	GGTCTGGTGTCAAAAATTAG CAGCTTAATAATAACCGGG	Addition of LAA to *lacZ* in pSM128
LAAR	CCCGGTTATTATTAAGCTGC TAATTTTTGACACCAGACC	Addition of LAA to *lacZ* in pSM128
ASVF	GGTCTGGTGTCAAAAA GCATCAGTT TAATAATAACCGGG	Addition of ASV to *lacZ* in pSM128
ASVR	CCCGGTTATTATTAAACTGA TGCTTTTTGACACCAGACC	Addition of ASV to *lacZ* in pSM128
tail.LAAF	GGTCTGGTGTCAAAAAGCA GCAAACGACGAAAACTACG CTTTAGCAGCTTAATAATAA C	Addition of AADENYA to *lacZ* in pSM128
tail.LAAR	GTTATTATTAAGCTGCTAAA GCGTAGTTTTCGTCGTTTGC TGCTTTTTGACACCAGACC	Addition of AADENYA to *lacZ* in pSM128
tail.ASVF	GGTCTGGTGTCAAAAAGCA GCAAACGACGAAAACTACG CTGCATCAGTTTAATAATAA C	Addition of AADENYA to *lacZ* in pSM128
tail.ASVF	GTTATTATTAAACTGATGCA GCGTAGTTTTCGTCGTTTGC TGCTTTTTGACACCAGACC	Addition of AADENYA to *lacZ* in pSM128
SDM1F	GCGAATGTCAACGGTGTGG ACATATATCAGGG	SDM on putative -10 element from TGTGTT to GGTGTG in upstream region of Rv0560c
SDM1R	CCCTGATATATGTCCACACC GTTGACATTCGC	SDM on putative -10 element from TGTGTT to GGTGTG in upstream region of Rv0560c
SDM2F	CTGTGTTGACAGATAGCAG GGCCGTTG	SDM on putative -10 element from TATATC to GATAGC in upstream region of Rv0560c
SDM2R	CAACGGCCCTGCTATCTGT CAACACAG	SDM on putative -10 element from TATATC to GATAGC in upstream region of Rv0560c
SDM3F	CAGGGCCGTTGAGATATAG	SDM on putative -10 element from TATATAT to GATATAG in upstream

	GAACACATGAC	region of Rv0560c
SDM3R	GTCATGTGTTCCTATATCTC AACGGCCCTG	SDM on putative -10 element from TATATAT to GATATAG in upstream region of Rv0560c
SDMpro1F	CAGGGCCGTCCATATATAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to CCATATATAT in upstream region of Rv0560c
SDMpro1R	GTCATGTGTTCATATATATG GACGGCCCTG	SDM on extended -10 element from TGATATATAT to CCATATATAT in upstream region of Rv0560c
SDMpro2F	CAGGGCCGTCGATATATAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to CGATATATAT in upstream region of Rv0560c
SDMpro2R	GTCATGTGTTCATATATATC GACGGCCCTG	SDM on extended -10 element from TGATATATAT to CGATATATAT in upstream region of Rv0560c
SDMpro3F	CAGGGCCGTTCATATATATG AACACATGAC	SDM on extended -10 element from TGATATATAT to TCATATATAT in upstream region of Rv0560c
SDMpro3R	GTCATGTGTTCATATATATG AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TCATATATAT in upstream region of Rv0560c
SDMpro4F	CAGGGCCGTTGATGTATAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to TGATGTATAT in upstream region of Rv0560c
SDMpro4R	GTCATGTGTTCATATACATC AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TGATGTATAT in upstream region of Rv0560c
SDMpro5F	CAGGGCCGTTGATAGATAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to TGATAGATAT in upstream region of Rv0560c
SDMpro5R	GTCATGTGTTCATATCTATC AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TGATAGATAT in upstream region of Rv0560c
SDMpro6F	CAGGGCCGTTGATATGTAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to TGATATGTAT in upstream region of Rv0560c
SDMpro6R	GTCATGTGTTCATACATATC AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TGATATGTAT in upstream region of Rv0560c
SDMpro7F	CAGGGCCGTTGATATAGAT GAACACATGAC	SDM on extended -10 element from TGATATATAT to TGATATAGAT in upstream region of Rv0560c
SDMpro7R	GTCATGTGTTCATCTATATC AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TGATATAGAT in

		upstream region of Rv0560c
SDMpro8F	CAGGGCCGTTGATATATGT GAACACATGAC	SDM on extended -10 element from TGATATATAT to TGATATATGT in upstream region of Rv0560c
SDMpro8R	GTCATGTGTTCACATATATC AACGGCCCTG	SDM on extended -10 element from TGATATATAT to TGATATATGT in upstream region of Rv0560c
SDMO1F	CTGTGTTGACAGGTATCAG GGCCGTTG	SDM on operator region from TATATCAGGGCCGT to GGTATCAGGGCCGT in upstream region of Rv0560c
SDMO1R	CAACGGCCCTGATACCTGT CAACACAG	SDM on operator region from TATATCAGGGCCGT to GGTATCAGGGCCGT in upstream region of Rv0560c
SDMO3F	CTGTGTTGACATATAGGAG GGCCGTTG	SDM on operator region from TATATCAGGGCCGT to TATAGGAGGGCCGT in upstream region of Rv0560c
SDMO3R	CAACGGCCCTCCTATATGTC AACACAG	SDM on operator region from TATATCAGGGCCGT to TATAGGAGGGCCGT in upstream region of Rv0560c
SDMO4F	GTTGACATATATCGCGGCC GTTGATATATATG	SDM on operator region from TATATCAGGGCCGT to TATATCGCGGCCGT in upstream region of Rv0560c
SDMO4R	CATATATATCAACGGCCGC GATATATGTCAAC	SDM on operator region from TATATCAGGGCCGT to TATATCGCGGCCGT in upstream region of Rv0560c
SDMO5F	GTTGACATATATCAGCCCCG TTGATATATATG	SDM on operator region from TATATCAGGGCCGT to TATATCAGCCCCGT in upstream region of Rv0560c
SDMO5R	CATATATATCAACGGGGCTG ATATATGTCAAC	SDM on operator region from TATATCAGGGCCGT to TATATCAGCCCCGT in upstream region of Rv0560c
SDMO6F	GTTGACATATATCAGGGGG GTTGATATATATG	SDM on operator region from TATATCAGGGCCGT to TATATCAGGGGGGT in upstream region of Rv0560c
SDMO6R	CATATATATCAACCCCCCTG ATATATGTCAAC	SDM on operator region from TATATCAGGGCCGT to TATATCAGGGGGGT in upstream region of Rv0560c

Appendix 6. Primers used for amplification of *lacZ* and *rpoB* for antisense plasmids.

*Hind*III and *BamH*I restriction sites are underlined.

Primer	Sequence (5' to 3')	Purpose
lacZH	CCCAAGCTTCCGTCGTTTTACAACGTCGTG	Amplification of *lacZ*
lacZB	CCCGGATCCTTATTTTTGACACCAGACCA	Amplification of *lacZ*
rpoBH	CCCAAGCTTCTCGTTGGTCGCATGAAGTGCTGG	Amplification of *rpoB*
rpoBB	CCCGGATCCGGGTTTAGTTTTGCGACAGCTTTACG	Amplification of *rpoB*

Appendix 7. Vectors and plasmids used for the study of upstream regions of antibitotic-responsive genes.

Plasmid name	Description	Source
pSC-A	PCR cloning vector, AmpR	Stratagene
pSM128	LacZ promoter probe vector, integrating, AmR	(Dussurget *et al.,* 1999)
pFLAME3	GFP promoter probe vector (LAA tag), replicative, KanR	(Blokpoel *et al.,* 2003)
pFLAME4	GFP promoter probe vector (ASV tag), replicative, KanR	(Blokpoel *et al.,* 2003)
pDS137	457 bp upstream region of Rv1015c in pSC-A	This study
pDS138	529 bp upstream region of Rv0277c in pSC-A	This study
pDS139	507 bp upstream region of *whiB6* in pSC-A	This study
pDS140	518 bp upstream region of Rv3898c in pSC-A	This study
pDS141	525 bp upstream region of Rv2487c in pSC-A	This study
pDS142	472 bp upstream region of *whiB7* in pSC-A	This study
pDS143	358 bp upstream region of Rv0748 in pSC-A	This study
pDS144	223 bp upstream region of Rv0608 in pSC-A	This study
pDS141	525 bp upstream region of Rv2487c in pSC-A	This study
pDS162	Upstream region of Rv0277c in pSM128	This study
pDS155	Upstream region of Rv0608c in pFLAME3	This study
pDS156	Upstream region of Rv0608c in pFLAME4	This study
pDS157	Upstream region of Rv0748c in pFLAME3	This study
pDS158	Upstream region of Rv0748c in pFLAME4	This study
pDS154	Upstream region of Rv1015c in pSM128	This study
pDS153	Upstream region of Rv2487c in pSM128	This study
pDS145	Upstream region of Rv3898c in pFLAME3	This study
pDS148	Upstream region of Rv3898c in pFLAME4	This study
pDS150	Upstream region of *whiB6* in pFLAME3	This study
pDS152	Upstream region of *whiB6* in pFLAME4	This study

pDS159	Upstream region of *whiB7* in pFLAME3	This study
pDS160	Upstream region of *whiB7* in pFLAME4	This study
pDS134	376 bp upstream region of Rv2487c in pSC-A	This study
pDS135	542 bp upstream region of Rv1015c in pSC-A	This study
pDS204	433 bp upstream region of *whiB6* in pSC-A	This study
pDS168	New upstream region of Rv2487c in pSM128	This study
pDS170	New upstream region of Rv1015c in pSM128	This study
pDS207	New upstream region of *whiB6* in pSM128	This study

Appendix 8. Plasmids for the study of upstream regions of hypoxia-responsive genes.

Plasmid name	Description	Source
pDS3	254 bp upstream region of *hspX* in pSC-A	This study
pDS5	356 bp upstream region of Rv2625c in pSC-A	This study
pDS7	328 bp upstream region of Rv2627c in pSC-A	This study
pDS29	438 bp upstream region of Rv2626c in pSC-A	This study
pDS129	180 bp upstream region of Rv2625c in pSC-A	This study
pDS17	Upstream region of Rv2625c in pSM128	This study
pDS36	Upstream region of Rv2626c in pSM128	This study
pDS53	Upstream region of Rv2627c in pSM128	This study
pDS81	Upstream region of *hspx* in pSM128	This study
pDS125	Upstream region of Rv2625c in pSM128 with putative -10 element mutated from CTTTAT to CTTTGC	This study
pDS131	180 bp upstream region of Rv2625c in pSM128	This study
pDS163	Upstream region of Rv2625c in pSM128 with putative -10 element mutated from TCTACT to TCTGCC	This study

Appendix 9. Plasmids used for the study of upstream regions of salicylate-responsive genes.

Plasmid name	Description	Source
pDS132	660 bp upstream region of Rv0560c in pSC-A	This study
pDS133	690 bp upstream region of Rv0561c in pSC-A	This study
pDS167	pSM128 with upstream region of Rv0560c	This study
pDS169	pSM128 with upstream region of Rv0561c	This study
pDS175	pSM128 with ASV tag on *lacZ*	This study
pDS176	pSM128 with LAA tag on *lacZ*	This study
pDS180	Upstream region of Rv0560c in pSM128 with putative -10 element mutated from TGTGTT to GGTGTG	This study
pDS181	Upstream region of Rv0560c in pSM128 with putative -10 element mutated from TATATC to GATAGC	This study
pDS182	Upstream region of Rv0560c in pSM128 with putative -10 element mutated from TATATAT to GATATAG	This study
pDS183	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to CCATATATAT	This study
pDS184	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to CGATATATAT	This study
pDS185	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to TCATATATAT	This study
pDS186	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to TGATGTATAT	This study
pDS187	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to TGATAGATAT	This study
pDS188	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to TGATATGTAT	This study
pDS189	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT to TGATATAGAT	This study
pDS190	Upstream region of Rv0560c in pSM128 with extended -10 element mutated from TGATATATAT	This study

	to TGATATATGT	
pDS194	Upstream region of Rv0560c in pSM128 with ASV tag on *lacZ*	This study
pDS195	Upstream region of Rv0560c in pSM128 with LAA tag on *lacZ*	This study
pDS211	Upstream region of Rv0560c in pSM128 with operator region mutated from TATATCAGGGCCGT to GGTATCAGGGCCGT	This study
pDS212	Upstream region of Rv0560c in pSM128 with operator region mutated from TATATCAGGGCCGT to TATAGGAGGGCCGT	This study
pDS213	Upstream region of Rv0560c in pSM128 with operator region mutated from TATATCAGGGCCGT to TATATCGCGGCCGT	This study
pDS217	Upstream region of Rv0560c in pSM128 with operator region mutated from TATATCAGGGCCGT to TATATCAGCCCCGT	This study
pDS214	Upstream region of Rv0560c in pSM128 with operator region mutated from TATATCAGGGCCGT to TATATCAGGGGGGT	This study

Appendix 10. Plasmids used for knockdown of *lacZ* and *rpoB*.

Plasmid name	Genotype or characteristics	Source
pMV206	Mycobacteria - *E. coli* shuttle cloning vector, replicative, KanR	(Stover *et al.,* 1991)
pDS171	328 bp upstream region of Rv2627c in pMV206	This study
pDS172	Upstream region of Rv2627c with antisense orientation *lacZ* in pMV206	This study
pDS173	Upstream region of Rv2627c with antisense orientation *rpoB* in pMV206	This study
pDS193	660 bp upstream region of Rv0560c in pMV206	This study
pDS198	Upstream region of Rv0560c with antisense oreintation *lacZ* in pMV206	This study
pDS199	Upstream region of Rv0560c with antisense orientation *rpoB* in pMV206	This study

Appendix 11. Composition of Middlebrook 7H9 and Dubos medium.

Component for 1 L	Middlebrook 7H9	Dubos
Ammonium sulphate	0.5 g	-
L-glutamic acid	0.5 g	-
Sodium citrate	0.1 g	-
Pancreatic digest of casein	-	0.5 g
L-asparagine	-	2.0 g
Polysorbate 80	-	0.2 g
Pyridoxine	1.0 mg	-
Biotin	0.5 mg	-
Disodium phosphate	2.5 g	2.5 g
Monopotassium phosphate	1.0 g	1.0 g
Ferric ammonium citrate	0.04 g	50 mg
Magnesium sulphate	0.05 g	10 mg
Calcium chloride	0.5 mg	0.5 mg
Zinc sulphate	1.0 mg	0.1 mg
Copper sulphate	1.0 mg	0.1 mg

Appendix 12. Chemical structures of compounds tested for their effect on $P_{Rv0560c}$ activity.