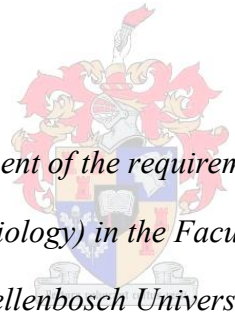
Regulation of efflux in rifampicin resistant mutants of *Mycobacterium tuberculosis*

by

Danicke Willemse



Thesis presented in partial fulfilment of the requirements for the degree Master of Science in Medical Science (Molecular Biology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr Monique Joy Williams

Co-supervisors: Prof Thomas Calldo Victor and Prof Robin Mark Warren

March 2013

AUTHOR'S DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Danicke Willemse

Date: March 2013

ABSTRACT

Multidrug resistant tuberculosis (MDR-TB), defined as having resistance to at least the first-line drugs, isoniazid and rifampicin (RIF), is a global health problem. Mutations in the *rpoB* gene, encoding the β-subunit of RNA polymerase, are implicated in RIF resistance - with the S531L and H526Y mutations occurring most frequently. The level of RIF resistance varies for strains with identical *rpoB* mutations, which suggests that other factors play a role in RIF resistance. Efflux has been implicated in determining the intrinsic level of RIF resistance. Increased expression of the multidrug efflux pump, *Rv1258c*, following RIF exposure was observed in some *Mycobacterium tuberculosis* MDR clinical isolates and H37Rv RIF mono-resistant mutants, but not others. The factors influencing the induction of *Rv1258c* are poorly understood.

The aim of this study was to investigate the effects of *rpoB* mutations on expression of *Rv1258c* and *whiB7*, a transcriptional regulator of *Rv1258c*, in *M. tuberculosis* H37Rv *in vitro* generated RIF resistant mutants, in the absence and presence of RIF.

The promoter region of *M. tuberculosis* H37Rv *Rv1258c* was cloned into a position upstream of a *lacZ* gene (encoding β-galactosidase) in multi-copy episomal and integrating vectors. Vector functioning and the effect of *rpoB* mutations on *Rv1258c* promoter activity were initially investigated in the non-pathogenic related species, *Mycobacterium smegmatis* mc^2155 *rpoB* mutants and subsequently in *M. tuberculosis* by doing β-galactosidase assays. qRT-PCR was done to investigate the effects of *rpoB* mutations on native *Rv1258c* and *whiB7* gene expression.

Episomal and integrating vectors were functional and the integrating vector system was used for subsequent β-galactosidase assays in *M. tuberculosis*. *Rv1258c* promoter activity in the S531L mutant was approximately 1.5 times less and in the H526Y mutant 1.5 times higher than that of the wild-type in *M. smegmatis*. Similarly, *Rv1258c* promoter activity in the S531L mutant was approximately half and in the H526Y mutant approximately double that of the wild-type in *M. tuberculosis*. A similar trend in *Rv1258c* and *whiB7* expression to those observed using β-galactosidase assays were observed when investigating the native *Rv1258c* and *whiB7* gene transcript levels compared to the wild-type using qRT-PCR, although

differences were not significant. Exposure of the *M. smegmatis* and *M. tuberculosis rpoB* mutants to sub-inhibitory levels of RIF did not affect *Rv1258c* promoter activity.

Mutations in *rpoB* had a marginal effect on *Rv1258c* and *whiB7* transcript levels, but showed the same trend as that seen for *Rv1258c* promoter activity. It remains to be determined whether these differences are biologically significant. When considering efflux pumps as new targets for treatment, possible differences in efflux pumps expression due to different *rpoB* mutations should be considered.

OPSOMMING

Multi-middel weerstandige tuberkulose (MDR-TB) word gedefinieer as weerstandigheid tot ten minste rifampisien (RIF) en isoniasied, wat deel van die eerstelyn anti-tuberkulose behandeling vorm. Mutasies in die *rpoB* geen, wat die β-subeenheid van die RNA polimerase enkodeer, word geassosieer met RIF weerstandigheid. S531L en H526Y *rpoB* mutasies kom die algemeenste voor. RIF weerstandigheids vlakke verskil egter tussen isolate met identiese *rpoB* mutasies, wat impliseer dat ander faktore ook ‘n rol in RIF weerstandigheid speel. ‘n Toename in transkripsie van die *Rv1258c* geen, wat ‘n multi-middel effluks pomp enkodeer, is waargeneem met blootstelling aan RIF, slegs in sommige *M. tuberculosis* H37Rv RIF mono-weerstandige mutante and MDR kliniese isolate, maar nie in ander nie. Die faktore wat die induksie van die *Rv1258c* effluks pomp beïnvloed is nie goed nagevors nie.

Die studie ondersoek die effek van die *rpoB* mutasies op die uitdrukking van die *Rv1258c* en *whiB7*, ‘n transkripsionele regulator van *Rv1258c*, gene in *M. tuberculosis* H37Rv *in vitro* gegenereerde RIF weerstandige mutante, in die teenwoordigheid en afwesigheid van RIF.

Die promotor area van die *M. tuberculosis* H37Rv *Rv1258c* geen is in ‘n posisie stroomop van ‘n *lacZ* geen, wat vir β-galaktosidase enkodeer, in multi-kopie episomale en integreerende vektors ingekloneer. Die funksionaliteit van die vektor en effek van *rpoB* mutasies op *Rv1258c* promotor aktiwiteit is ondersoek in die naverwante nie-patogeniese spesies, *M. smegmatis* en daarna in *M. tuberculosis* deur β-galaktosidase essais te doen. qRT-PCR is gedoen om die effek van *rpoB* mutasies op die vlak van transkripsie van die natuurlike *Rv1258c* geen en die *whiB7* geen te bestudeer.

Beide die episomale en integreerende vektors was funksioneel en daar is besluit om die integreerende vektor vir daaropeenvolgende β-galaktosidase essais in *M. tuberculosis* te gebruik. *Rv1258c* promotor aktiwiteit van die S531L mutant was ongeveer 1.5 keer minder as en die van die H526Y mutant 1.5 keer hoër as die van die ongemuteerde bakterië in *M. smegmatis*. Soortgelyk was die *Rv1258c* promoter aktiwiteit van die S531L mutant ongeveer die helfde van en die van H526Y mutant ongeveer dubbel die van die ongemuteerde bakterië in *M. tuberculosis*.

‘n Soortgelyke neiging in die vlakke van *Rv1258c* en *whiB7* transkripte van die natuurlike geen is gedurende qRT-PCR waargeneem alhoewel die verskille nie beduidend was nie. Blootstelling aan sub-inhibitoriese konsentrasies van RIF het geen effek op *Rv1258c* uitdrukking in die *M. smegmatis* of *M. tuberculosis rpoB* mutante gehad nie.

Die *rpoB* mutasies het net ‘n effense effek op *Rv1258c* en *whiB7* transkrip vlakke in *M. tuberculosis rpoB* mutante, maar transkrip vlakke het ‘n soortgelyke neiging as die *Rv1258c* promoter aktiwiteit getoon. Of die waargenome verskille biologies betekenisvol is, moet nog bepaal word. Indien effluks pompe as teikens vir bahandeling gebruik sou word, moet in ag geneem word dat effluks pompe moontlik verskillend uitgedruk word in verskillende *rpoB* mutante.

ACKNOWLEDGEMENTS

I would like to acknowledge the following people and institutions for their contribution to this study:

- My supervisor Dr Monique Williams for her help, support, encouragement and believe in my ability to successfully complete this research. Also, for teaching me many laboratory skills, thoughtful discussion of my work and critical reading of my thesis.
- My co-supervisors Prof TC Victor and Prof RM Warren for their help and thoughtful discussion of my work as well as reading and commenting on my thesis.
- Anastasia Koch at the University of Cape Town for providing the *M. smegmatis* mc^2155 *rpoB* mutants.
- Margaretha de Vos for help generating the *M. tuberculosis* H37Rv *rpoB* mutants and for teaching me how to determine the minimum inhibitory concentrations (MICs) for the *M. tuberculosis rpoB* mutants using the MGIT Bactec system.
- Marieta McGrath for help generating the *M. tuberculosis* H37Rv *rpoB* mutants.
- Melanie Grobbelaar for teaching me how to do RNA extractions, helping me with experion chip runs and answering questions regarding RNA work.
- Dr Sven Parsons for his help with qRT-PCR work and thoughtful discussions of the results.
- My mother (Marlene), father (Wikus) and sister (Lodene) for their unwavering support, encouragement, love and help.
- The DST/NRF Centre of Excellence in Biomedical Tuberculosis Research at Stellenbosch University for providing the research funding for this project.
- Harry Crossley Grants for providing research funding for this project.
- DAAD-NRF in Country Scholarship and Ernst and Ethel Eriksen trust for bursaries provided, making it possible for me to carry on with my studies.
- Last, but not least, I want to thank the Lord for providing me with the intellectual capacity to be able to study and for guiding me and providing me with strength, perseverance and help to complete this research.

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ADDENDUMS

Page

LIST OF ABBREVIATIONS

ACH	Acyl-homoserine lactone
acrAB	Acridine AB
aph	Aminoglycoside phosphotransferase gene
ATP	Adenosine triphosphate
β	Beta
β-gal	Beta-galactosidase
bp	Base pairs
B. pseudomallei	*Burkholderia pseudomallei*
BSA	Bovine serum albumin
B. subtilis	*Bacillus subtilis*
cAMP	3',5' cyclic adenosine monophosphate
CCCP	Carbonyl cyanide *m*-chlorophenylhydazone
cDNA	Complementary deoxyribonucleic acid
°C	Degrees celcius
CFU	Colony forming units
C. jejuni	*Campylobacter jejuni*
CRP	cAMP receptor protein
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Dr	Doctor
E. coli	*Escherichia coli*
far	Fatty acid resistance gene
Fig	Figure
Fur	Ferric uptake regulator
g	Gram
gfp	Green fluorescent protein encoding gene
GU	Growth units
gyr	Gyrase gene
HAQ	4-hydroxy-2-alkylquinoline
HHQ	4-hydroxy-2-heptylquinoline
kbp	Kilo base pairs
lacZ	β-galactosidase encoding gene
LB	Luria-Bertani
Mar	Multiple antibiotic resistance
MATE	Multidrug and toxic compound extrusion
M. avium	*Mycobacterium avium*
M. chelonae	*Mycobacterium chelonae*
MCS	Multiple cloning site
MDR	Multidrug resistant

M. fortuitum	*Mycobacterium fortuitum*
MFS	Major facilitator superfamily
$MgCl_2$	Magnesium chloride
MICs	Minimum inhibitory concentrations
min	Minutes
ml	Mililitre
mm	Milimetre
mM	Milimolar
M. marinum	*Mycobacterium marinum*
mRNA	Messenger RNA
M. smegmatis	*Mycobacterium smegmatis*
Mtr	Multiple transferrable resistance
M. tuberculosis	*Mycobacterium tuberculosis*
ng	Nonogram
N. gonorrhoeae	*Neisseria gonorrhoeae*
nm	Nono metre
OADC	Oleic acid-dextrose-catalase
OD	Optical density
ONPG	O-nitrophenyl-β-D-galactopyranoside
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
P. putida	*Pseudomonas putida*
PQS	Pseudomonas Quinolone Signal
Prof	Professor
qRT-PCR	Quantitative real time PCR
RACE	Rapid amplification of cDNA ends
RE	Restriction enzyme
RIF	Rifampicin
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
RRDR	Rifampicin resistance determining region
rRNA	Ribosomal RNA
s	Seconds
S	Svedburg units
S. aureus	*Staphylococcus aureus*
S. lividans	*Streptomyces lividans*
SdiA	Suppressor of division inhibition
SigA	Sigma factor A
S. maltophilia	*Stenotrophomonas maltophilia*
SMR	Small multidrug resistance
T	Terminator
TB	Tuberculosis
Tm	Annealing temperature
Tris	Tris(hydroxymethyl)aminomethane

Tris-EDTA	Tris-EDTA buffer
ttg	Toluene tolerance operons
Tween 80	Polyoxyethylene sorbitan monooleate
U	Units
V	Volt
WCLs	Whole cell lysates
WHO	World Health Organization
X-gal	5-Bromo-4 Chloro-3 Indolyl β-D-galactosidase
Zn	Ziehl-Neelsen
µ	Micro
µF	MicroFarad
Ω	Ohm
×g	Centrifugal force

CHAPTER 1: LITERATURE REVIEW: REGULATION OF MULTIDRUG EFFLUX PUMPS

Currently, drug-resistance in many clinically relevant human pathogens is a major problem worldwide. This is because of the adaptation of these pathogens and acquisition of numerous mechanisms that make them largely resistant to treatment with antibiotics and a wide range of other antimicrobial agents (Grkovic *et al.*, 2002). Adaptation is necessary in order for bacteria to survive in the harsh environment that they have been exposed to over many years. Bacteria are constantly challenged by exposure to antibiotics, disinfectants and antiseptics that are widely used (Grkovic *et al.*, 2001). Pathogenic bacteria will become increasingly resistant to these compounds if we do not elucidate and gain a complete understanding of the mechanisms of drug resistance as well as regulation and interconnectedness of these mechanisms, in order to effectively counteract them. Some mechanisms that are used by bacteria to evade the toxic effect of drugs include: drug inactivation and modification, using alternate metabolic pathways to overcome the effects of antimicrobial drugs on certain pathways, altering or mutating drug target sites and preventing drug entry into the cell by using permeability barriers or loss of porins as well as active efflux of drugs (Nikaido, 1998; Putman *et al.*, 2000; Grkovic *et al.*, 2002).

Efflux has for long been implicated in drug resistance in bacteria, but has only recently been implicated in rifampicin (RIF) (Gupta *et al.*, 2006; Louw *et al.*, 2011), isoniazid (Srivastava *et al.*, 2010), ethambutol (Gupta *et al.*, 2006; Srivastava *et al.*, 2010), streptomycin (Gupta *et al.*, 2006), ofloxacin (Gupta *et al.*, 2006; Singh *et al.*, 2011), ethidium bromide, novobiocins and pyrroles (Balganesh *et al*., 2010) resistance in *Mycobacterium tuberculosis*. *M. tuberculosis* is the causative agent of the devastating disease, tuberculosis (TB). The development of drug resistance of this pathogen to almost all known antituberculosis drugs has necessitated research into the discovery of new drug targets. The finding that efflux pumps are involved in drug resistance in *M. tuberculosis* has lead to efflux pumps being considered as targets for drug development. One strategy involves the use of efflux pump inhibitors in combination with existing or new drugs as it was shown to increase susceptibility to the drugs (Balganesh *et al*., 2010; Louw *et al*., 2011; Balganesh *et al*., 2012).

Efflux pumps are present in gram-positive and gram-negative bacteria. Some of these pumps consist of a single protein present in the cytoplasmic membrane while other gram-negative

bacterial efflux pumps have three membrane components across the cytoplasmic and outer membranes (Nikaido, 1998). Efflux pumps are divided into phylogenetic groups based on transport mode, energy source, structure, protein family, distribution of homologues in other organisms and substrate specificity (Saier *et al*., 1998; Putman *et al*., 2000; Van Bambeke *et al*., 2000). Primary active efflux pumps typically use energy from ATP hydrolysis as energy source while secondary active efflux pumps use the electrochemical proton or sodium ion gradients across the membrane as energy source (Putman *et al.*, 2000; Van Bambeke *et al*., 2000). The ATP binding cassette (ABC) superfamily efflux pumps are primary active efflux pumps and are phylogenetically similar based on topology, protein family, energy source and distribution of homologues (Saier *et al*., 1998).

The secondary active efflux pumps are further divided into the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) family and the multidrug and toxic compound extrusion (MATE) family based on their primary and secondary structures (Putman *et al.*, 2000). The MFS efflux pumps are further divided into families based on sequence similarity where members within a family are homologous and these phylogenetic families correlate to function (Pao *et al*., 1998). The SMR and RND families are further divided into phylogenetic subfamilies based on their function (Saier *et al*., 1998). The MATE family efflux pumps are the most recently categorized (Kurodo & Tsuchiya, 2009).

Some efflux pumps only extrude a specific drug or class of drugs whereas others can extrude a wide range of structurally unrelated compounds (multidrug efflux pumps) (Putman *et al*., 2000). MDR efflux pumps have been identified in all of the efflux pump families (Pao *et al*., 1998; Saier *et al*., 1998; Van Bambeke *et al*., 2000; Kurodo & Tsuchiya, 2009). Genes encoding multidrug efflux pumps are regulated at both local and global level by prokaryotic transcriptional regulators. These regulators are classified into families based on sequence similarity, structure and function (Ramos *et al.*, 2005).

Local transcriptional regulation provides regulation of a specific efflux pump adjacent or in close proximity the local regulator, thus providing a more confined transcriptional effect (Grkovic *et al.*, 2001) while global transcriptional regulation simultaneously alters the expression of several genes located at different positions in the genome. This review will mainly focus on the regulation of efflux at local and global level with examples of this

regulation in various bacteria including mycobacteria. The induction of efflux pumps by drugs they export, role of pH, metabolic energy and ions in modulating efflux pump function, regulation of efflux pumps by quorum sensing and physiological roles of efflux pumps are addressed.

1.1 LOCAL REGULATION OF MULTIDRUG EFFLUX PUMPS

1.1.1 Regulation by the drugs exported via the local regulators

In many cases, multidrug efflux pumps are induced by antibiotics they export. This induction occurs via local regulators of the efflux pumps. In this case, drugs interact directly with a regulator, which in turn alters expression of the efflux system. Examples of regulation of the efflux pump expression by their substrates are present in many bacterial species including mycobacteria. As an easy to follow guideline, Table 1.1 summarises the local regulators, efflux pumps they regulate and compounds by which they are induced that are discussed in this review.

The induction of the *smeDEF*-encoded efflux pump is implicated in resistance to several antibiotics and biocides including triclosan in *Stenotrophomonas maltophilia*. The SmeT repressor regulates *smeDEF* gene expression (Hernández *et al.*, 2009; Hernández *et al.*, 2011). In the absence of an inducer, SmeT binds to a 28 bp palindromic sequence that includes the promoters of both *smeT* and *smeDEF*, preventing transcription of both genes (Hernández *et al.*, 2009; Hernández *et al.* 2011). Two triclosan molecules bind to the SmeT repressor (which is a homodimer) and causes stabilization of SmeT in a conformation unable to bind to DNA. This prevents binding of SmeT to the *smeDEF* promoter and de-represses *smeDEF* transcription (Hernández *et al.*, 2009; Hernández *et al.*, 2011). The efflux pump substrate is therefore responsible for the induction of efflux pump expression.

The TtgR repressor in *Pseudomonas putida* regulates *ttgABC* efflux pump expression, which is one of three toluene tolerance operons (ttg) including *ttgABC*, *ttgDEF* and *ttgGHI* present in *P. putida* (Rojas *et al.*, 2003). The *ttgR* gene is situated adjacent to and divergently transcribed from the *ttgABC* operon (Duque *et al.*, 2001). A *ttgR* null mutant in which the promoters of the *ttgR* and *ttgA* genes were still intact showed increased *ttgA* and *ttgR* promoter expression confirming that TtgR acts as repressor of the *ttgABC* efflux system as

well as its own expression (Duque *et al.*, 2001). In the absence of substrates of the TtgABC efflux system, the TtgR repressor is bound to a palindromic operator site overlapping both the *ttgABC* and *ttgR* promoters in the *ttgR*/*ttgABC* intergenic region, presumably blocking RNA polymerase access to the *ttgABC* and *ttgR* promoters (Terán *et al*., 2003). In the presence of chloramphenicol and tetracycline, TtgR dissociates from the operator leaving the promoters of *ttgABC* and *ttgR* exposed for transcription and induction of both genes occurs. Induction of *ttgABC* and *ttgR* expression by antibiotics is dose dependent and mediated by the induction of the regulator itself. Interestingly, the *ttgABC* and *ttgR* genes are expressed at basal levels even in the absence of these antibiotics, suggesting that its induction is regulated by other molecules and suggesting a possible physiological function (Terán *et al*., 2003).

The TtgR repressor has two different overlapping drug binding sites. One is broader with hydrophobic residues while the other is deeper with polar residues. These different binding sites allows for some versatility in binding to ligands, which is why TtgR is able to bind to many molecules with dissimilar structures, sizes, shapes and charge. This allows for the induction of the *ttgABC* efflux pump system by a diverse range of molecules including antibiotics and plant antimicrobials (phloretin, quercetin and naringenin) (Alguel *et al.*, 2007).

Interestingly, although the TtgR repressor is a member of the TetR family of repressors, it has unique drug binding sites to that of the TetR repressor after which the TetR family of regulators are named. TetR binds only to tetracycline, while TtgR binds to multiple compounds. Binding of tetracycline to TtgR is distinct in that the position in which it fits into the binding site is different to TetR (Alguel *et al.*, 2007). Tetracycline binds to the TetR repressor with its amino groups on the inside of the binding pocket and its cyclic ring towards the outside of the pocket while it binds to TtgR with its amide groups at the bottom of the binding pocket and cyclic rings towards the outside. TtgR also shows strong structure similarity to QacR family of transporters in *Staphylococcus aureus* discussed later (Alguel *et al.*, 2007). This indicates how efflux pump regulators, which are classified into the same family, do not necessarily display the same ligand binding properties even for the same ligand.

TtgV is a repressor encoded in the *ttgVW* operon, which is adjacent to the divergently transcribed *ttgGHI* operon (Rojas *et al.*, 2003; Guazzoni *et al.*, 2007). Deletion of *ttgV* caused

an increase in expression of *ttgGHI* and *ttgVW*, while deletion of *ttgW*, had no effect on *ttgGHI* and *ttgVW* expression. TtgV thus acts as a repressor of the *ttgGHI* and *ttgVW* genes, regulating the pump expression and its own. TtgV binds to the *ttgGHI*/*ttgVW* intergenic region containing the promoters of both operons (Rojas *et al.*, 2003). In the absence of an effector, TtgV binds to the DNA in a tetrameric state and with a high affinity causing bending of the operator DNA that presumably blocks RNA polymerase binding to the *ttgGHI* and *ttgVW* promoters (Guazzaroni *et al.*, 2007). Interestingly, when different mutations were introduced in the operator of TtgV, it caused either an increase or decrease in *ttgGHI* and *ttgVW* expression. Although these mutations have not been observed clinically, it is tempting to speculate that undiscovered mutations might occur in the operator regions of efflux pump regulators and could lead to an additional level of efflux pump regulation that is clinically relevant (Guazzarroni *et al.*, 2004).

The TtgGHI efflux pump in *P. putida* is induced by aromatic hydrocarbons, antibiotics and aliphatic alcohols (Rojas *et al.*, 2003; Guazzaroni *et al.*, 2004). Binding of aliphatic alcohols to TtgV causes dissociation of TtgV from the operator in the intergenic region, and exposes the promoters of *ttgGHI* and *ttgVW* so that transcription can occur (Guazzaroni *et al.*, 2004). The tetrameric state of TtgV is maintained in the absence and presence of an inducer (Guazzaroni *et al.*, 2007). In contrast to most members of the IclR family of transcriptional regulators, TtgV is not as specific in the compounds it binds to and subsequently displays multidrug binding properties (Guazzaroni *et al.*, 2007). Although there is a high degree of homology (70%) at protein level between the TtgABC and TtgGHI efflux systems, they have different substrate specificities (Terán *et al.*, 2003), indicating that minor changes in the efflux pumps at protein level can significantly affect substrate binding properties.

The *bpeR* gene, encoding the BpeR repressor, is divergently transcribed from the *bpeAB-oprB* operon, encoding a multidrug efflux pump in *Burkholderia pseudomallei* (Chan *et al.*, 2004; Chan & Chua, 2005). Overexpression of *bpeR* results in repression of *bpeA* (Chan & Chua, 2005). Investigation of the MIC for numerous drugs in a *bpeAB* deletion mutant, revealed increased susceptibility to the aminoglycosides gentamicin and streptomycin and the macrolide erythromycin relative to the wild-type implicating BpeAB-OprB in export of these drugs and a multi-drug resistance phenotype (Chan *et al.*, 2004). Erythromycin induces the expression of both *bpeAB* and *bpeR*, demonstrating that a substrate of this pump could mediate its induction via the BpeR regulator (Chan & Chua, 2005).

In some organisms, the repressor and efflux genes are encoded in the same operon. In *S. aureus* a large multi-resistance plasmid encodes QacR in the same open reading frame as *qacA,* and *qacB* (encoding a closely related protein to QacA from which QacA is thought to have evolved) (Grkovic *et al.*, 2003). In the absence of toxic compounds transported by QacA, QacR represses *qacA* expression by binding to a palindromic sequence, inverted repeat 1 (IR1) overlapping the *qacA* promoter (Grkovic *et al.*, 1998). QacA exports several chemically distinct antimicrobial compounds. Many dissimilar compounds, including plant alkaloids, presumably cause a conformational change in QacR and subsequent release of QacR from the promoter region, allowing *qacA* expression (Grkovic *et al.*, 1998; Grkovic *et al.*, 2003).

In *Escherichia coli* the *emrR* gene is located upstream of the *emrA* gene in the *emrRAB* operon. Overexpression of *emrR* caused decreased expression of the *emrRAB* operon and mutations in *emrR* caused overexpression of *emrRAB*, indicating that EmrR is a repressor of the *emrRAB* operon (Lomovskaya *et al.*, 1995). In the absence of inducers, EmrR binds to the transcriptional start site of the *emrRAB* operon and represses *emrRAB* operon transcription (Xiong *et al.*, 2000; Grkovic *et al.*, 2001). Exposure to several structurally unrelated compounds that are EmrA substrates including nalidixic acid and carbonyl cyanide *m*-chlorophenylhydazone (CCCP), interfered with binding of EmrR to its operator and thus caused a EmrR-dependent induction of the *emrRAB* genes (Xiong *et al.*, 2000).

The *acrR* gene in *E. coli* encodes the AcrR repressor and is located 141 bp upstream of and divergently transcribed from the *acrAB* (acridine AB) efflux pump. The AcrB efflux pump is responsible for the efflux of different toxic compounds in *E. coli* (Su *et al.*, 2007). In the absence of inducers, AcrR binds to a 28 bp operator, between the *acrR* and *acrAB* genes, which overlaps with the *acrAB* promoter, thereby repressing transcription of the efflux pump encoding gene *acrB* (Su *et al.*, 2007). Some of the pump substrates bind to AcrR and cause dissociation of AcrR from the promoter region thereby de-repressing the efflux system. AcrR can bind to two drugs simultaneously (Su *et al.*, 2007).

Induction of efflux pumps by the compounds they export via the local regulator is dependent on the drug concentration. The CmeR transcriptional repressor in *Campylobacter jejuni* belongs to the TetR family and is encoded on the same strand upstream of the *cmeABC* multidrug RND efflux pump (Lin *et al.*, 2005a). In the absence of an inducer, CmeR binds to

the inverted repeat region between the *cmeR* and *cmeA* genes, preventing transcription. Mutations in this region caused reduced binding of CmeR, increased expression of *cmeABC* and decreased susceptibility to antibiotics including erythromycin, ciprofloxacin and norfloxacin. CmeR is thus a repressor of *cmeABC* (Lin *et al.*, 2005a).

CmeR is a dimeric molecule consisting of two domains and has a large flexible ligand binding pocket. The conformation of CmeR changes when ligands bind to it (Gu *et al.*, 2007). Sub-lethal concentrations of most of the antibiotics exported by CmeABC including erythromycin and ciprofloxacin, could however not induce the efflux pump expression (Lin *et al.*, 2005b). Since induction of some efflux pumps is dose-dependent, higher substrate concentrations than those tested may be required for induction (Terán *et al.*, 2003).

In *Streptomyces lividans*, EbrA, EbrB and EbrC are efflux pumps thought to be involved in ethidium bromide resistance, since their overexpression conferred elevated resistance to this compound. The *ebrS* gene encodes a transcriptional regulator of the AcrR family and regulates expression of *ebrC*. The *ebrS* gene is present upstream of *ebrC* and is divergently transcribed. EbrS represses the transcription of *ebrC* and itself. Overexpression of *ebrS* therefore leads to increased repression of *ebrC*, resulting in ethidium bromide sensitivity. Conversely, when *ebrS* was deleted, elevated ethidium bromide resistance was observed due to *ebrC* induction (Lee *et al.*, 2007). Eight drugs, including six known substrates of EbrC (tetracycline, nalidixic acid, methyltriphenylphosphonium, proflavin, norfloxacin and ethidium bromide) failed to induce *ebrC* expression, while RIF and calcium chloride induced the *ebrS* promoter resulting in *ebrC* repression. Calcium ions are hypothesized to bind directly to EbrS and prevent self-repression (Lee *et al.*, 2007).

Increased transcription of a pump may be facilitated by enhanced binding of a transcriptional activator in the presence of an efflux pump substrate. An example of this is the regulation of Bmr, a multidrug transporter in *Bacillus subtilis*, by the transcriptional activator, BmrR. Expression of *bmr* is induced by two structurally diverse compounds rhodamine 6G and tetraphenylphosphonium (TPP) which are exported by Bmr. Dimerised BmrR binds to the *bmr* promoter in the absence of the ligand, but its binding is enhanced in the presence of the substrates resulting in induction of *bmr* (Ahmed *et al.*, 1994). Some aromatic cationic compounds also bind to BmrR resulting in induction of *bmr* expression (Markham *et al.*, 1997). The ability of BmrR to recognize diverse compounds with different structures, allows

the induction of the *bmr* by multiple drugs. When determining the crystal structure of BmrR bound to tetraphenylphosphonium, it was established that during formation of a complex of the drug and BmrR, the multidrug-binding domain of an alpha helix of BmrR unfolds and relocates. Subsequently, this shift causes an internal drug binding pocket to be exposed, to which the drug then binds (Zheleznova *et al.*, 1999). This multidrug binding pocket has a redundant negative nature and is rigid allowing for some control over which substrates induce the expression of the multidrug efflux pump Bmr and which do not (Newberry *et al.*, 2008). The binding pocket can also solvate drugs with a range of different conformations, which all bind in the same orientation to the binding pocket (Bachas *et al.*, 2011). This illustrates how multiple drugs with diverse structures are able induce efflux pump expression through their ability to bind to a single transcriptional regulator.

1.1.2 Local regulation of multidrug efflux pumps in mycobacteria

LfrA is a multidrug efflux pump implicated in the efflux of fluoroquinolones, cationic dyes and anthracyclines in *M. smegmatis* (Liu *et al.*, 1996; Buroni *et al.*, 2006). The *lfrR* gene is encoded upstream of *lfrA* and the genes are co-transcribed from a common promoter. LfrR acts as a transcriptional repressor of the *lfrRA* operon. In the absence of LfrA substrate, LfrR binds to the promoter of *lfrRA* and completely represses transcription of *lfrRA* (Buroni *et al.*, 2006). Ciprofloxacin and acriflavine, substrates of the LfrA efflux pump, bind to LfrR and cause the release of LfrR from the promoter region of *lfrRA* and in the process de-represses l*frA* expression. The drugs themselves thus induced the expression of the *lfrA* efflux pump, which exports them (Buroni *et al.*, 2006).

Crystallographic and calorimetric studies have shown the intrinsic flexibility of the LfrR transcriptional repressor in *M. smegmatis*. This flexibility is thought to provide the protein with the ability to bind to many different drugs and undergo conformational changes in order to de-repress the efflux pumps (Bellinzoni *et al.*, 2009). Interestingly, two binding reactions were shown to occur for LfrR with proflavine and ethidium bromide. Which binding reaction occurs, depends on the proflavine/LfrR concentration ratio. The first binding reaction occurs with a high affinity while the second binding reaction has a much lower binding affinity. At a low molar ratio of ethidium bromide/LfrR, one ethidium bromide molecule binds with high affinity and at a high molar ratio the ethidium bromide molecule binds with an even higher affinity to LfrR. This shows that the release of LfrR repression and subsequent induction of

Table 1.1. Summary of the local regulators and efflux pumps they regulate as well as which drugs induce efflux pump expression via interaction with the local regulator.

Efflux pump system	Present in	Local regulator	Family of transcriptional regulators	Efflux pump induced specifically via the local regulator in the presence of	References
smeDEF (RND pump)	*S. maltophilia*	SmeT repressor	TetR	Biocide (trisclosan)	(Hernández *et al.*, 2009; Hernández *et al.*, 2011)
ttgABC (RND pump)	*P. putida*	TtgR repressor (shows structure similarity to QacR family of regulators in *S. aureus*)	TetR	Antibiotics (chloramphenicol and tetracycline) and plant antimicrobials (phloretin, quercetin and naringenin)	(Duque *et al.*, 2001; Rojas *et al.*, 2003; Terán *et al.*, 2003; Alguel *et al.*, 2007)
ttgGHI (RND pump)	*P. putida*	TtgV repressor	IclR	Aliphatic alcohols (1-hexanol and 1-naphthol) and nitrotoluene	(Rojas *et al.*, 2003; Guazzaroni *et al.*, 2004; Guazzaroni *et al.*, 2007)
bpeAB-oprB (RND pump)	*B. pseudomallei*	BpeR repressor	TetR	Erythromycin	(Chan *et al.*, 2004; Chan & Chua, 2005)
qacRAB (MFS pump)	*S. aureus*	QacR repressor	TetR	Plant alkaloids, diamidines, guanidines and dyes	(Grkovic *et al.*, 1998; Grkovic *et al.*, 2003)
emrRAB (MFS pump)	*E. coli*	EmrR repressor	MarR	Nalidixic acid, CCCP; 2,4-dinitrophenol and tetrachlorosalicylanilide	(Lomovskaya *et al.*, 1995; Xiong *et al.*, 2000; Grokovic *et al.*, 2001)

Continued overleaf

Table 1.1. Summary of the local regulators and efflux pumps they regulate as well as which drugs induce efflux pump expression via interaction with the local regulator (continued).

Efflux pump system	Present in	Local regulator	Family of transcriptional regulators	Efflux pump induced specifically via the local regulator in the presence of	References
acrAB	*E. coli*	AcrR repressor	TetR	Ethidium, proflavin and rhodamine 6G	(Su *et al.*, 2007)
bmr (MFS pump)	*B. subtilis*	BmrR activator	MerR	Rhodamine and TPP and lipophilic aromatic cationic compounds	(Ahmed *et al.*, 1994; Markham *et al.*, 1997; Zheleznova *et al.*, 1999; Newberry *et al.*, 2008; Bachas *et al.*, 2011)
lfrRA (MFS pump)	*M. smegmatis*	LfrR repressor	TetR	Ciprofloxacin, acriflavine, proflavine and ethidium bromide	(Buroni *et al.*, 2006; Bellinzoni *et al.*, 2009)
mmr (SMR pump)	*M. tuberculosis*	Rv3066 repressor	TetR	Ethidium bromide	(Bolla *et al.*, 2012)

the efflux pump is dependent on the concentration of the drug. Higher concentration of drug might be needed to induce efflux (Bellinzoni *et al.*, 2009).

The Mmr multidrug efflux pump exports multiple drugs including acriflavine, ethidium bromide and erythromycin in *M. tuberculosis* (De Rossi *et al.*, 1998). The *rv3066* gene is present immediately downstream of the *mmr* gene. In the absence of compounds extruded by Mmr, Rv3066 is bound to IR1 of the *mmr* promoter and causes repression of *mmr* transcription. Ethidium bromide binds to the multidrug-binding pocket of the Rv3066 regulator causing a conformational change and dissociation of the Rv3066 repressor from the *mmr* promoter occurs. There is thus a drug-mediated de-repression of the *mmr* gene expression and transcription of *mmr* occurs (Bolla *et al.*, 2012).

1.2 GLOBAL TRANSCRIPTIONAL REGULATION OF MULTIDRUG EFFLUX PUMPS

Global transcriptional regulators enable bacteria to control the expression of multiple genes simultaneously, facilitating a concomitant response to environmental cues such as exposure to antibiotics, nutrient starvation and other environmental stresses.

1.2.1 Global transcriptional regulation of efflux in bacteria other than mycobacteria

In addition to regulation of the *acrAB* efflux system in *E. coli* by the local regulator AcrR, this pump is also regulated by a global regulator, MarA (multiple antibiotic resistance). (Cohen *et al.*, 1993). The *marA* gene encodes the global transcriptional activator, MarA, belonging to the XylS/AraC family (Alekshun & Levy, 1997). MarA induces a number of genes encoding proteins of diverse functions including genes present in the *marRAB* operon as well as the *acrA* gene which forms part of the *acrAB* efflux system discussed earlier (Barbosa & Levy, 2000). *MarR* encodes a local transcriptional repressor of the *marRAB* operon and represses transcription when no inducers are present (Cohen *et al.*, 1993; Seoane & Levy, 1995). MarR repression of the *marRAB* operon is reversed by the presence of compounds that are involved in stress response (Seoane & Levy, 1995).

Another example of global regulation of efflux is provided in the regulation of the *bmr* gene in *B. subtilis* which is not only regulated by the local regulator BmrR as discussed earlier, but

also regulated by a global regulator, Mta, which belongs to the MerR family of regulators. Mta, like BmrR, binds to the *bmr* promoter and induces or activates the transcription of *bmr*. It also induces transcription of the *blt* gene which encodes another multidrug efflux pump, Blt. It is not known which compounds bind to Mta to induce it, although Mta has been shown to auto-regulate its own expression (Baranova *et al.*, 1999). Mta is thus a global transcriptional activator.

The MtrCDE (multiple transferrable resistance) multidrug resistance efflux pump in *Neisseria gonorrhoeae* belongs to the RND family and exports macrolide antibiotics, penicillin and other antimicrobials. A local repressor of the TetR family, MtrR and a global transcriptional activator belonging to the AraC/XylS-type activators, MtrA, regulate the MtrCDE efflux pump system (Hagman & Shafer, 1995; Rouquette *et al.*, 1999; Lee *et al.*, 2003). The *mtrR* gene is encoded 250 bp downstream of the *mtrCDE* operon and is divergently transcribed (Hagman & Shafer, 1995). MtrR binds to the region between *mtrR* and *mtrC*, which includes the *mtrCDE* and *mtrR* promoter regions (Lucas *et al.*, 1997). Mutations in the MtrR binding region causes increased *mtrCDE* expression and decreased *mtrR* expression, indicating that MtrR is a repressor of the *mtrCDE* efflux pump (Lucas *et al.*, 1997). Mutations in the *mtrR* gene and its promoter region have also been identified in clinical isolates of *N. gonorrhoeae* and are associated with increased resistance to penicillin, erythromycin and azithromycin (Warner *et al.*, 2007). MtrA plays a role in the induction of *mtrCDE* by sub-lethal concentrations of pump substrates. It is not known whether this regulation of *mtrCDE* by MtrA occurs directly or indirectly (Rouquette *et al.*, 1999). The *mtrCDE* efflux pump system is thus positively and negatively regulated by a global activator and local repressor as summarized in Fig 1.1.

Interestingly, *mtrR* mutations, which cause de-repression of the *mtrCDE* efflux pump system, also lead to a significant increased fitness of the mutant bacteria relative to wild-type bacteria in a mouse infection model. This increased fitness was maintained throughout the course of infection, and was specifically due to de-repression of the MtrCDE efflux pump system (Warner *et al.*, 2007). When comparing the relative *in vivo* fitness of a *mtrA* mutant with that of the wild-type in *N. gonorrhoeae*, a fitness cost was seen within two days of inoculation (in a mouse infection model) in the *mtrA* mutant compared to the wild-type. Interestingly, spontaneous compensatory *mtrR* mutations were selected in *mtrA* mutants during infection,

demonstrating that the expression levels of the *mtrCDE* efflux system have a significant impact on the fitness of *N. gonorrhoeae* (Warner *et al.*, 2007).

In addition to the role of MtrR as local regulator of the *mtrCDE* efflux pump, MtrR is also a global regulator as it controls the expression of about 70 genes across the genome. MtrR indirectly regulates the expression of the *farAB*-encoded efflux pump via regulation of a local regulator, FarR. The *farAB* (fatty acid resistance) operon consists of the membrane fusion protein, FarA, and the cytoplasmic membrane transporter protein, FarB, belonging to the MFS superfamily. This efflux pump uses the MtrE protein as the outer membrane channel to extrude long-chain antibacterial fatty acids and is required for resistance to antibacterial fatty acids (Lee *et al.*, 2003). At the local level, *farAB* is regulated by transcriptional repressor, FarR, which binds directly to the *farAB* promoter and causes transcriptional repression of the efflux system. MtrR binds to the *farR* promoter, repressing *farR* expression and causing de-repression of the *farAB* efflux system. MtrR therefore not only functions as a negative regulator of the *mtrCDE* efflux system, but also as an indirect positive regulator for *farAB* efflux system (Lee *et al.*, 2003). MtrR and FarR-mediated regulation also extends to genes involved in other aspects of metabolism. For example, FarR acts as a transcriptional activator of *glnA*, which encodes glutamine synthetase. MtrR therefore indirectly decreases *glnA* expression by reduced binding of FarR to its promoter. The regulation of *mtrCDE* and *farAB* by MtrR is illustrated in Fig 1.1. This demonstrates that global regulation often involves a cascade of events, which can simultaneously induce or repress the expression of genes with diverse functions within the cell (Johnson *et al.*, 2011).

NorA is a MDR efflux pump of the MFS family that exports a variety of drugs such as fluoroquinolones, ethidium bromide, cetrimide, benzalkonium chloride, tetraphenylphosphonium bromide and acrifavine in *S. aureus* (Fournier *et al.*, 2000). The *norR* gene is present upstream of *norA* and overexpression of *norR* led to increased *norA* expression and increased resistance to quinolones and ethidium bromide. NorR is thus a local activator or positive regulator of the *norA* efflux pump (Truong-Buldoc *et al.*, 2003). The *arlR-arlS* genes encode regulatory genes with ArlR being a possible regulatory protein and ArlS a histidine kinase (Fournier *et al.*, 2000). The *norA* gene expression is negatively regulated by the ArlR/S regulator (Fournier *et al.*, 2000). The ArlR/S regulator has its effects on *norA* expression by modulating (decreasing) NorR binding to the *norA* operator (Truong-Buldoc *et al.*, 2003). The *norA* gene is negatively regulated by the global regulator MgrA

(Luong *et al.*, 2006). MgrA is a global transcriptional regulator that shares homology with MarR in *E. coli*. MgrA activates the transcription of many genes and acts a repressor of many others (Luong *et al.*, 2003; Luong *et al.*, 2006). MgrA acts as negative regulator of two other efflux systems, *norB* and *tet38* (Truong-Buldoc *et al.*, 2005).

1.2.2 Global transcriptional regulation of efflux in mycobacteria

The MSMEG_2173 global regulator in *M. smegmatis* belongs to the GntR/FadR family of transcription factors. MSMEG_2173 binds to a specific palindromic DNA sequence and searching the genome of *M. smegmatis* for this specific sequence revealed 292 potential target genes with binding sites for MSMEG_2173. These genes included a broad range of thirty-seven transporter genes and this was confirmed with immunoprecipitation with an MSMEG_2173 antibody. Deletion of *MSMEG_2173* in *M. smegmatis* increased expression of most of the genes with predicted binding sites. Conversely, overexpression of *MSMEG_2173* decreased expression of the same genes. This is indicative that MSMEG_2173 acts as a negative regulator, repressing the expression of multiple transporter genes (Rao *et al.*, 2012). MSMEG_2173 was also implicated in negatively regulating drug transporters, since overexpression of *MSMEG_2173* caused increased susceptibility to isoniazid (MIC of 160 µg/ml). A strain overexpressing *MSMEG_2173* grew poorly in 30 µg/ml isoniazid, while the wild-type strain had a MIC of 200 µg/ml. Deletion of *MSMEG_2173*, increased the MIC for isoniazid to 300 µg/ml and the mutant showed enhanced growth in 30 µg/ml isoniazid (Rao *et al.*, 2012).

Lsr2 is a histone-like protein that is highly conserved among mycobacteria and acts as a transcriptional repressor of the *iniBAC* efflux pump. The iniBAC pump is induced by isoniazid and ethambutol. The expression of *iniBAC* promoter was elevated and ethambutol resistance increased in a *M. smegmatis lsr2* mutant relative to the wild-type. This is indicative of a role of Lsr2 repressor in *iniBAC* expression (Colangeli *et al.*, 2007). Deletion of *lsr2* in *M. smegmatis* resulted in increased expression of *iniA* and other genes involved in cell wall and metabolic functions, indicating a role of Lsr2 in repressing a broad range of genes including those not involved in efflux (Colangeli *et al.*, 2007). Overexpression the *lsr2* gene in *M. tuberculosis* resulted in a down-regulation of the isoniazid-mediated induction of the *iniBAC* genes, indicating the role of Lsr2 in *M. tuberculosis* gene repression (Colangeli *et al.*, 2007). Also, overexpression of *lsr2* in *M. tuberculosis* lead to a down-regulation of the

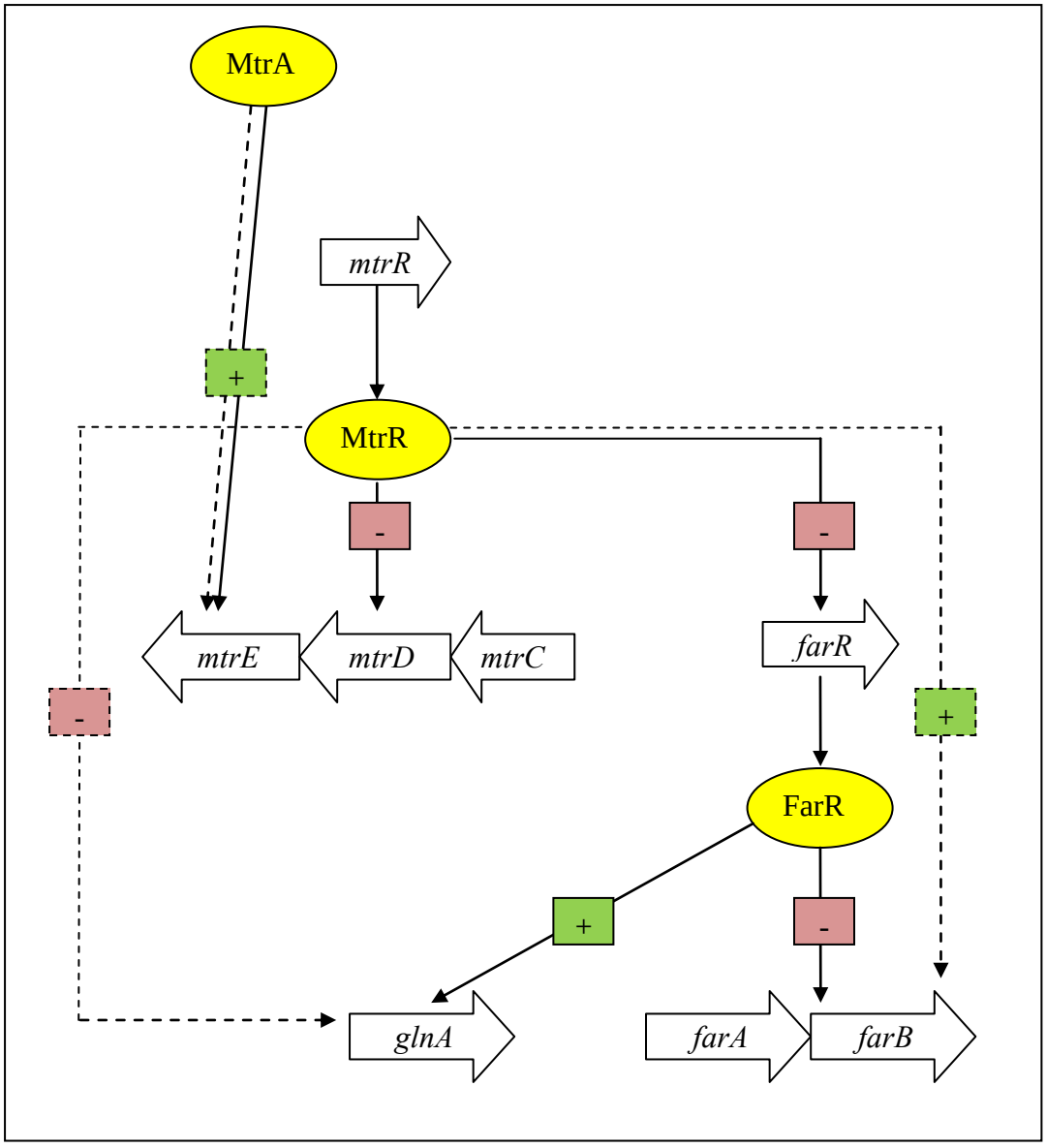


Figure 1.1. Regulation of efflux pumps by MtrA, MtrR and FarR regulators. Solid lines represent direct regulation, dashed lines indirect regulation and solid and dashed lines together indicate that the mechanism of regulation is unknown.

isoniazid-mediated induction of *efpA*, another putative efflux pump (Colangeli *et al.*, 2007) which is part of the QacA family of transporters and is present in several slow and fast growing mycobacterial species (Doran *et al.*, 1997). Thus, Lsr2 has a possible role in the global regulation of multiple efflux pump systems as well as other genes not involved in efflux in mycobacteria. This shows how histone-like protein can also act as global regulators.

1.2.3 The WhiB family of global transcriptional regulators in *M. tuberculosis*

The WhiB family of genes in *M. tuberculosis* is a family of key global transcriptional regulators that bind to DNA and interact with proteins causing activation of transcription. They are present only in actinomycetes (Geiman *et al.*, 2006; Burian *et al.*, 2012). These regulators will be discussed in more detail since they are relevant to the current study. *M. tuberculosis* has seven *whib*-like genes that are structurally and functionally different, making each of the WhiB proteins a distinguished member of the family (Alam *et al.*, 2009). Sequence analysis of WhiB proteins revealed four conserved cysteine residues, two of which are present in a CXXC motif. WhiB proteins also contain redox-sensitive iron-sulphur clusters (Alam *et al.*, 2009), which are versatile cofactors that participate in electron transfer, substrate binding or activation, iron or sulphur storage, gene expression regulation and enzyme activity (Alam *et al.*, 2007). The seven WhiB proteins in *M. tuberculosis* are thought to have distinct functions, and are involved in physiological processes such as cell division, reacting to nutrient starvation, pathogenesis and stress sensing, as well as antibiotic resistance. Mutations in the different *whiB* genes cause distinct phenotypes and the *whiB* mutants react differently to stress conditions (Alam *et al.*, 2009).

The redox potential of WhiB1 matches that of thioredoxin-like proteins, and its ability to catalyze the reduction of insulin disulfide demonstrates that it functions as a disulfide reductase (Garg *et al.*, 2007). The promoter of *whiB1* contains a cAMP receptor protein (CRP) binding site. *whiB1* expression is regulated by cAMP levels through cAMP-dependent binding of CRP. In the presence of cAMP (at high enough levels), CRP binds to the *whiB1* promoter inducing its transcription (Agarwal *et al.*, 2006). WhiB2 has chaperone properties as it restored the function of a chemically denatured protein and suppressed the aggregation of proteins independent of its redox state and in the absence of ATP in *M. tuberculosis* H37Rv (Konar *et al.*, 2012). WhiB3 maintains the intra-bacterial redox homeostasis by regulating virulence lipid anabolism in response to the oxido-reductive stress encountered during macrophage infection (Singh *et al.*, 2009).

The apo mWhiB4 protein also shows similarity to thiorodoxin proteins and functions as a disulfide reductase following destruction of the iron sulphur (4Fe-4S) cluster in an oxidizing environment. It was therefore hypothesized that WhiB4 plays a role in the response to oxidative stress (Alam *et al.*, 2007). Indeed, WhiB4 expression was repressed by oxidative

stress and induced by antioxidants. Deletion of *whiB4* from *M. tuberculosis* caused changes in redox balance, a reduction of the membrane potential, increased *in vitro* oxidative stress resistance and the mutant over-expressed the *M. tuberculosis* antioxidant system, indicating the role of WhiB4 in regulating oxidative stress response in *M. tuberculosis*. The knockout mutant was also hypervirulent indicating a role of WhiB4 in modulating virulence (Chawla *et al.*, 2012).

WhiB5 regulates the expression of 58 genes as identified by microarray. A *whiB5* null mutant was attenuated during infection and could not grow after reactivation. WhiB5 could also play a part in immunomodulation since there was a larger inflammatory response observed in mice infected with the *whiB5* knockout mutant than to those infected with the wild-type. WhiB5 is thus important in virulence, immunomodulation and reactivation as well as regulating many genes (Casonato *et al.*, 2012).

Geiman *et al.* (2006) investigated the expression of the *whiB*-like genes in *M. tuberculosis* following exposure to different stress conditions, including exposure to anti-tuberculosis drugs. Exposure to isoniazid (INH) increased *whiB2* and slightly decreased *whiB4* expression, while having minimal effects on the rest of the gene family. The expression of all of the genes was only minimally affected by ethambutol, except for an increased expression of *whiB2*. Kanamycin caused induction of *whiB2*, *whiB3*, *whiB6* and *whiB7*. Cycloserine increased the expression of *whiB2* and slightly decreased *whiB5*, *whiB6* and *whiB7* expression. Streptomycin caused induction of *whiB3*, *whiB6* and *whiB7* and repression of *whiB2* and *whiB5* (Geiman *et al.*, 2006). *M. tuberculosis whiB7* was induced by subinhibitory concentrations of antibiotics including erythromycin, tetracycline and streptomycin in a dose dependent manner and fatty acids which accumulate during host infection. Null mutants of *whiB7* were hypersusceptible to the aforementioned antibiotics (Morris *et al*., 2005). Microarray analyses, used to identify the genes in the *whiB7* regulon, showed that initially only *whiB7* was induced upon antibiotic exposure making it the primary regulator of the genes which were subsequently induced by antibiotic exposure. The genes which were induced by antibiotic exposure specifically through *whiB7* induction included *Rv1258c*, encoding a multidrug efflux pump, and *Rv1473*, encoding a putative macrolide transporter (Morris *et al*., 2005).

M. tuberculosis is known to infect and replicate within macrophages. Adams *et al.* (2011) showed that drug tolerant bacteria were present soon after infection and these drug tolerant bacteria are able to survive within macrophages. The intracellular environment encountered by *M. tuberculosis* within macrophages induces drug tolerance, which is mediated by the induction of efflux pumps. *Rv1258c* is induced upon macrophage infection and its induction mediates RIF tolerance. An *Rv1258c* knockout mutant of *M. tuberculosis* showed impaired survival in macrophages, indicating the importance of Rv1258c in intracellular growth as well as RIF resistance (Adams *et al.*, 2011). WhiB7 thus functions as a global transcriptional regulator and plays a role in drug resistance (Morris *et al.*, 2005).

The *WhiB7* promoter is conserved across actinomycetes (Burian *et al.*, 2012). Investigation of the promoter and structural gene sequence of *whiB7* in multidrug resistant and extensively drug resistant clinical isolates in *M. tuberculosis* showed a high degree of sequence conservation, indicating the importance of the WhiB7 proteins in *M. tuberculosis* (Arjomandzadegan *et al.*, 2011). Induction of *whiB7* by erythromycin was much lower in the *whiB7* mutant providing evidence that WhiB7 activates its own promoter. Burian *et al.* (2012) investigated the mechanism by which WhiB7 responds to antibiotics and activates drug resistance genes. *WhiB7* was induced by eighty-six structurally unrelated compounds demonstrating that its induction is not due to recognition of common structural motif. Instead, redox state was shown to control the level of *whiB7* induction, as *whiB7* was activated by a reduced environment. This shows that WhiB7 links physiology and antibiotic resistance by maintaining the redox potential and activating resistance genes at the same time (Burian *et al.*, 2012).

A novel regulator in *M. tuberculosis*, Rv2034, belongs to the ArsR family of regulators. Rv2034 auto-regulates its own expression by binding to its own promoter and repressing its own expression. A palindromic sequence, representing the Rv2034 binding sequence was identified. Inspection of the genome of *M. tuberculosis* for this sequence identified binding sites for Rv2034 in the promoter regions of multiple genes, including *whiB7*, indicating a possible role for Rv2034 in regulation of *whiB7* expression. Rv2034 also positively regulates the *dosR* gene that encodes DosR protein that acts as a key regulator of hypoxic response in *M. tuberculosis* (Gao *et al.*, 2012). This indicates how global regulators interact to regulate multiple regulons simultaneously.

1.3 ROLE OF PH, IONS AND METABOLIC ENERGY IN MODULATION OF EFFLUX PUMP EXPRESSION

In addition to regulation of efflux pumps at both local and global level, efflux pumps can also be modulated by pH and availability of ions and metabolic energy. The AcrAB-TolC efflux system in *Salmonella Enteridis serovar typhimurium* belongs to the RND family and exports ethidium bromide (Amaral *et al.*, 2011). The accumulation of ethidium bromide was shown to be less at a low pH (pH 5) as appose to a higher pH (pH 8), suggesting that the efflux system was more efficient at a low pH. At a pH of 7.4 or higher, metabolic energy (provided in the form of glucose) is necessary for the effective functioning of the efflux pumps in order to reduce the amount of ethidium bromide accumulated (Amaral *et al.*, 2011). Ions are also important in the functioning of the efflux systems since in the absence of ions, metabolic energy was required even at a low pH for the effective functioning of the efflux system (Amaral *et al.*, 2011). This could be because there is a high availability of protons (ions) to use for antiport (manifesting itself as proton motive force) at a low pH. While at a high pH, the metabolism provides the protons needed for antiport and metabolic energy is thus required for efflux pump functioning. The pH thus modulates the degree to which metabolic energy and ions are needed for efflux pump functioning (Amaral *et al.*, 2011).

The same scenario was true for *E. coli* where ethidium bromide was extruded more effectively at a lower pH (pH 5) as opposed to a higher pH and metabolic energy was needed for efflux of the drug at high pH (pH 8) (Martins *et al.*, 2009). It was suggested that two different efflux systems in *E. coli* are activated at a low and high pH respectively. At low pH, the efflux systems driven by proton motive force are activated while at high pH the efflux systems driven by ATP hydrolysis are activated (Martins *et al.*, 2009). Calcium ions were also shown to play a role in efflux in *E. coli* (Martins *et al.*, 2011).

The expression of *norA* in *E. coli* is not only regulated by the local regulator NorR and the global regulators ArlR/S and MgrA as discussed earlier, but the expression of *norA* is also iron responsive, more specifically, iron uptake was shown to repress *norA* expression (Deng *et al.*, 2012). Fur (ferric uptake regulator) is a master iron uptake regulator that activates genes to ensure iron acquisition and storage under conditions of iron deprivation. A *fur* deletion mutant showed a decreased expression of *norA* compare to the wild-type strain as well as two fold reduction in the MIC for norfloxacin. This shows that *fur* acts as a positive

regulator of *norA* and demonstrates how the expression of efflux pumps is also regulated by the environmental conditions such as iron availability. It was suggested that the physiological role of NorA involves siderophores secretion and not drugs secretion, explaining the link between the iron levels and *norA* expression (Deng *et al.*, 2012).

In *M. tuberculosis* copper ions was shown to cause inhibition of the previously described MSMEG_2173 regulator binding to DNA; resulting in the formation of an inactive DNA binding protein. There was a decrease in MSMEG_2173/DNA complex with an increase in copper concentration. Consequently, copper ions could partially reverse MSMEG_2173 function (Rao *et al.*, 2012). MSMEG_2173 does however have a higher binding affinity for DNA than for copper ions. The MSMEG_2173 regulator can thus in part be regulated by the presence of metal ions, a finding that might also be relevant to other global regulators.

1.4 REGULATION OF EFFLUX PUMPS BY QUORUM SENSING

Quorum sensing is the process by which bacteria communicate inter-cellularly by secreting chemical signals, which in turn regulate the expression of specific genes in the surrounding bacteria (Rahmati *et al.*, 2002). Quorum sensing has been implicated in regulation of efflux pump expression.

In the Singapore strain, KHW, of *B. pseudomallei*, expression of *bpeAB-OprB* efflux system is growth phase dependent as it increases gradually from mid-log growth to stationary phase. The regulator of *bpeAB-OprB,* BpeR, which was discussed above, is induced at early stationary phase (Chan & Chua, 2005). Since autoinducers were shown to cause induction of the *bpeAB* efflux system at early exponential phase as opposed to mid-log phase (advancing the same level as expression obtained after 24 hours by about 12 hours), it is hypothesized that the BpeAB-OprB efflux system might be regulated by quorum sensing in *B. pseudomallei* KHW (Chan & Chua, 2005). SdiA (suppressor of division inhibition) regulates cell division in a quorum-sensing dependent manner in *E. coli* and acts as a transcriptional activator. An *sdiA* null mutant had decreased expression of AcrB protein and was less resistant to fluoroquinolones compared to wild-type bacteria. SdiA positively regulates the *acrAB* efflux system since subsequent overproduction of SdiA caused an increase in protein levels of AcrA and AcrB and returned quinolone resistance (Rahmati *et al.*, 2002). Quorum-sensing may thus result in regulation of efflux pumps.

1.5 POSSIBLE ROLE OF EFFLUX IN BIOFILM FORMATION AND QUORUM SENSING

Many bacteria form biofilms. Biofilms are complex communities of bacteria that are metabolically and spatially structured and encased in an exopolysaccharide matrix and are located at a phase interface. These complex structures can consist of a single or multiple bacterial species (Mah & O'Toole, 2001; Nikolaev & Plakunov, 2007). Bacteria that are part of a biofilm express different properties than planktonic bacteria and are metabolically heterogeneous, grow at different rates, experience different environmental conditions and are more resistant to most antimicrobials even when there is no genetic basis for resistance (Mah & O'Toole, 2001; Stewart & Costerton, 2001; Nikolaev & Plakunov, 2007).

When comparing gene expression of *E. coli* biofilms and planktonic bacteria, many genes encoding efflux pumps, known to be involved in efflux of toxic compounds including antibiotics, were shown to be highly upregulated during biofilm growth. Efflux pump inhibitors also reduce biofilm formation in different bacteria. Combinations of efflux pump inhibitors with different mechanisms of action synergistically decreased biofilm formation, suggesting that a variety of efflux pumps are involved in biofilm formation (Kvist *et al.*, 2008). Deletion of the genes encoding a putative efflux pump in a *Pseudomonas aeruginosa* biofilm leads to an increased drug sensitivity only in bacteria growing in a biofilm, but not for planktonic bacteria and is thus indicates biofilm specific drug resistance presumably brought about by efflux (Zhang & Mah, 2008).

Biofilms form in numerous mycobacterial species including *Mycobacterium fortuitum*, *Mycobacterium chelonae Mycobacterium avium*, *Mycobacterium marinum* and *M. smegmatis* (Hall-Stoodley & Lappin-Scott, 1998; Bardouniotis *et al.*, 2003; Yamazaki *et al.*, 2006; Nguyen *et al.*, 2010). While *M. tuberculosis* forms biofilms *in vitro*, (Ojha *et al.*, 2008) it is only speculated that biofilms might form *in vivo* after escape of the bacteria from phagosomes (Ojha & Hatfull, 2012). Biofilm dependent biocide resistance occurs in *M. fortuitum* biofilms (Bardouniotis *et al.*, 2003) and biofilm dependent RIF and isoniazid resistance occurs in *M. tuberculosis* (Ojha *et al.*, 2008). It is tempting to speculate that there could possibly be an undiscovered role for efflux pumps in biofilm drug resistance in *M. tuberculosis* as indicated in other bacteria.

Results of studies to determine the importance of efflux pumps in biofilms drug resistance are contradictory. In *P. aeruginosa* the expression of multidrug efflux pumps, *MexAB-OprM* and *MexCD-OprJ,* were decreased during biofilm formation in *P. aeruginosa* (De Kievit *et al.*, 2001). Hyperexpression of *mexCD-oprJ* in biofilm forming bacteria did not increase antibiotic resistance and in a *mexAB-OprM* mutant, biofilms still had high level resistance compared to planktonic cells indicating that these pumps did not contribute to the innate antibiotic resistance of biofilms (De Kievit *et al.*, 2001). MDR efflux pumps that have been implicated in drug resistance in planktonic *P. aeruginosa*, do thus not play a role in biofilm drug resistance (De Kievit *et al.*, 2001). However, resistance to ofloxacin, but not ciprofloxacin was shown to depend on *mexAB-OprM* expression in the biofilm, but only at low concentrations of ofloxacin (Brooun *et al.*, 2000). A maximal activity of MexAB-OprM and MexCD-OprJ were observed at the biofilm substratum (De Kievit *et al.*, 2001).

Efflux pumps also play a role in quorum sensing. The MexEF-OprN efflux pump in *P. aeruginosa* exports 4-hydroxy-2-heptylquinoline (HHQ) which is a 4-hydroxy-2-alkylquinoline (HAQ) autoinducer and is a Pseudomonas Quinolone Signal (PQS) precursor (Lamarche & Déziel, 2011). Autoinducers are homoserine lactones that accumulate in the growth medium depending on the cell density. When critical levels are reached, certain genes within the bacteria are induced, resulting in a population-wide response (Evans *et al.*, 1998). The MexEF-OprN efflux pump thus indirectly plays a role in quorum sensing by efflux of PQS that is involved in quorum sensing (Lamarche & Déziel, 2011). The MexAB-OprM efflux system extrudes an autoinducer, homoserine lactone autoinducer (PAI-1), which is involved in expression of virulence factors in *P. aeruginosa*. Overexpression of *mexAB-oprM* also causes a decline in autoinducer production. (Evans *et al.*, 1998).

A *bpeAB* efflux pump mutant in *B. pseudomallei* KHW was defective in the secretion of acyl-homoserine lactones (Chan *et al.*, 2007). In a *bpeR*-overexpressing strain, autoinducer production did not occur which shows how the BpeR repressor overexpression can inhibit quorum sensing (Chan & Chua, 2005). BpeRAB-OprB was also necessary for siderophore and phospholipase C production. The latter two compounds are quorum-sensing-controlled virulence factors (Chan & Chua, 2005). Additionally, BpeAB-OprB is also involved in biofilm formation as biofilm formation was significantly reduced in a *bpeAB* deletion mutant and in a *bpeR*-overexpressing strain. The BpeR regulator and BpeAB-OprB efflux pump are thus important in autoinducer production, quorum sensing, virulence factor production as

well as biofilm formation in *B. pseudomallei* KHW (Chan & Chua, 2005). Interestingly, in another strain, *B. pseudomallei* 1026b, BpeAB-OprB did not play a role in acyl-homoserine lactone (ACH) export or influence siderophore production, but was still necessary for biofilm formation, indicating the effects of strain genetic background on efflux pump function (Mima & Schweizer, 2010).

The above studies show that the role of efflux pumps in autoinducer secretion and quorum-sensing and proves how important it is to control the expression of multidrug efflux pumps since the overexpression might have important physiological implications. A role for efflux in quorum sensing in mycobacteria has however not been established.

1.6 THE PHYSIOLOGICAL FUNCTIONS OF EFFLUX PUMPS

While multidrug efflux pumps are important in drug resistance, it is believed that the actual function of the efflux pumps are not drug efflux, but that all of these pumps have physiological functions, which are in many cases still unknown (Putman *et al.*, 2000; Grkovic *et al.*, 2002). This is one reason why the regulation of efflux is so important since physiological functions could be affected in the case of incorrect regulation of efflux pumps. Some examples of possible physiological functions have been named previously in this review and a few are subsequently discussed.

Multidrug efflux pumps extrude a variety of compounds such as detergents, bile salts, organic solvents and ionophores and may in the process protect the membrane and energy state of the cell (Putman *et al.*, 2000). This is indicative of a physiological role for efflux pumps. The expression of *cmeABC* efflux pump in *C. jejuni* discussed earlier was significantly increased upon addition of numerous bile salts to the media, in a dose and time-dependent manner (Lin *et al.*, 2005b). Bile salts were shown to interact and bind to the CmeR repressor increasing expression of *cmeABC* and resistance to multiple antibiotics in the presence of bile salts. It was suggested that the CmeABC efflux system's primary function is providing bile salt resistance as appose to drug resistance (Lin *et al.*, 2005b). Similarly, an efflux system originally thought to extrude numerous drugs in *Vibrio cholerae* was shown to extruded bile and renamed, *breAB*. BreR acts as a negative regulator of *breAB* expression as well as regulates its own expression by binding to the *breAB* and *breR* promoters. Bile salts were

able to disrupt the binding of BreR to the promoter regions and subsequently caused induction of the *breAB* efflux system (Cerda-Maira *et al.*, 2008).

The overexpression of the Blt multidrug transporter of *B. subtilis* leads to an increased efflux of spermidine. The presence of the *blt* gene, which encodes for spermidine acetyltransferase, downstream of the *blt* multidrug transporter suggests that the physiological role of the Blt efflux pump is to extrude spermidine (Putman *et al.*, 2000).

M. bovis BCG strains with disrupted homologue of the gene encoding the Tap efflux pump grew slower and stopped growing prematurely in liquid cultures. However, when culturing on solid medium, this growth defect was reversed. The bacterial morphology also changed as the bacteria became elongated after sub-cultivation in liquid. Disruption of the *tap* efflux pump was found to affect gene expression of 18 genes during stationary phase. These genes included those involved in virulence, detoxification, adaptation, intermediary metabolism and respiration, conserved hypotheticals and cell wall and cell processing. The Tap efflux pump is thus important in many important physiological processes during stationary phase (Ramón-García *et al.*, 2012).

Since toxic product accumulation is a problem during stationary phase, the authors suggested a physiological role of the Tap efflux pump to be the removal of these toxic by-products. Examination of the genes up- and down-regulated upon disruption of the *tap* efflux pump, supports their hypothesis. Stress response genes such as Heat shock protein R (HspR) and *Rv2466c* (involved in oxidative stress) were up-regulated. *Rv2913c* a D-amino acid aminohydrolase, was upregulated, which suggests the involvement of the Tap efflux pump in clearing the D-amino acids, which are components of cell wall synthesis that accumulates during stationary phase. *whiB6* was up-regulated even though this gene was previously thought to be irrelevant for transition into stationary phase (Ramón-García *et al.*, 2012). Genes that were down-regulated included those involved in cell wall synthesis, sugar transport, recycling of amino sugars which are important energy source during stationary phase, import of sugars needed for mycothiol biosynthesis and sigma E which is essential for growth in macrophages. A role of the Tap efflux pump in mycobacterial virulence was also suggested since early secretory antigenic target-6 (ESAT-6), which is involved in antigen secretion, was repressed in the deletion mutant of the *tap* gene (Ramón-García *et al.*, 2012).

The Tap-like efflux pump in *M. fortuitum* extrudes tetracyclines and aminoglycosides (Ramón-García *et al.*, 2006; Louw *et al.*, 2009). The Tap efflux pump is not only present in Mycobacterial clinical isolates, but also in different Mycobacterial species collected from soil in grasslands. These species include *M. fortuitum*, *M. alvei* and *M. septicum*. The Tap efflux pump was present in only a percentage of tetracycline resistant strains and was also found in some tetracycline sensitive strains, demonstrating that the presence of the Tap efflux pump does not necessarily cause resistance. It would also suggest that the function of the Tap efflux pump is not only drug resistance, but that some clinically relevant pathogenic Mycobacteria and soil Mycobacteria, which are exposed to tetracycline present in soil, have adapted in such a way as to use the Tap efflux pump to efflux drugs such as tetracyclines (Kyselková *et al.*, 2012). These studies show the physiological role for the Tap efflux pump in addition to its role in drug efflux.

Rv1258c, is a Tap-like efflux pump of the MFS family in *M. tuberculosis* (a homologue of the Tap efflux pump discussed above). Expression of the *Rv1258c* homologue in *M. smegmatis*, resulted in increased tetracycline resistance and a decrease in the intracellular tetracycline levels. This demonstrates that Rv1258c is able to transport tetracycline and is implicated in subsequent tetracycline resistance (De Rossi *et al.*, 2002). A knockout mutant of *Rv1258c* in *M. tuberculosis* H37Rv showed increased susceptibility to gentamicin, spectinomycin and amikacin while hyperexpression decreased susceptibility to these drugs implicating *Rv1258c* in their efflux (Balganesh *et al*., 2012). Rv1258c is also implicated in RIF resistance as increased *Rv1258c* expression was observed following exposure to RIF in some MDR *M. tuberculosis* strains (Siddiqi *et al.*, 2004; Gupta *et al.*, 2006; Jiang *et al.*, 2008). The Rv1258c efflux pump in *M. tuberculosis* might also have physiological functions as the Tap efflux pump in *M. bovis* BCG discussed.

1.7 CONCLUSION

Multidrug efflux pumps are regulated by both local and global regulators. The local regulators are usually encoded in close proximity to the efflux pump that it regulates. The genetic arrangement of these regulators may vary, with some encoded upstream of the pump on the opposite strand, while others are present within the same operon as the efflux pump encoding gene. Either way, the local regulators regulate not only the efflux pump expression, but in some instances also their own expression.

Efflux pumps are often induced by the drugs that they export in a concentration dependent manner. This regulation occurs via binding of the drugs to the drug binding domains of the efflux pump local regulators that usually cause a conformational change. The conformational change in turn alters the regulator's affinity for the DNA and can cause either dissociation from the efflux pump promoter region resulting in de-repression (in the case of repressor) or can enhance binding to the promoter region activating transcription (in the case of activators).

In addition to local regulation, global regulators also regulate efflux pumps and provide the bacteria with the ability to control the expression of many sets of genes involved in different processes at once. The inclusion of efflux pumps in global regulated genes indicates the important contribution these efflux pumps to numerous processes. Efflux pump expression can also be modulated by pH, ions (for instance metal ions) and metabolic energy. Molecules involved in quorum-sensing have also been shown to regulate the expression of some efflux pumps.

Efflux pumps have physiological functions in addition to the drug efflux and it is believed that the drug efflux is actually a secondary acquired function of these pumps. This why regulation of efflux pumps is so important since accidental export of a needed compound can be detrimental to the bacteria. It is important that we gain a more complete understanding of the regulation of efflux since it is an important process contributing to drug resistance in many clinically relevant pathogenic species. Clearly the regulation of these pumps are complex and there are many levels to regulation, but understanding these regulatory processes might help us better combat drug resistant pathogens.

CHAPTER 2: STUDY RATIONAL, HYPOTHESES, AIMS AND EXPERIMENTAL DESIGN

2.1 STUDY RATIONAL

There were an estimated 8.7 million TB incident cases worldwide during 2011. MDR-TB, defined as having resistance to at least the first-line drugs, isoniazid and RIF, is a global health problem with 3.7% of new and 20% of previously treated TB cases globally estimated to have MDR-TB (World Health Organization (WHO), 2012).

RIF binds to the β-subunit of RNA polymerase forming a stable drug-enzyme complex, which causes premature termination of transcription and ultimately causes the death of bacteria (Johnson *et al.*, 2006). Central dogma suggests that RIF resistance is mainly associated with mutations in the 81 bp region encoding the β-subunit, called the RIF resistance determining region (RRDR) (Huitric *et al.*, 2006). Mutations such as single point mutations, insertions, deletions and multiple nucleotide changes in the *rpoB* gene are associated with RIF resistance. The S531L and H526Y *rpoB* mutations occur frequently in isolates from all over the world (Ohno *et al.*, 1996; Tracevska *et al.*, 2002; Huitric *et al.*, 2006; Madania *et al.*, 2012) and are associated with high level RIF resistance (Ohno *et al.*, 1996; Huitric *et al.*, 2006).

However, there is now growing evidence that efflux is also involved in determining the level of RIF resistance since exposure of *M. tuberculosis* MDR clinical isolates and RIF mono-resistant isolates to efflux pump inhibitors restored susceptibility to RIF (Louw *et al.*, 2011). Also, by using efflux pump inhibitors it was shown that RIF resistance could be mediated by an efflux mechanism (Gupta *et al.*, 2006). However, exposure of pan-susceptible strains to efflux pump inhibitors did not modulate RIF resistance (Louw *et al.*, 2011).

Several studies have implicated Rv1258c, a multidrug efflux pump, in RIF and other drug resistance in *M. tuberculosis* (Siddiqi *et al.*, 2004; Gupta *et al.*, 2006; Jiang *et al.*, 2008; Sharma *et al.*, 2010; Louw *et al.*, 2011). *Rv1258c* expression was increased upon exposure to RIF in an *in vitro* generated *M. tuberculosis* H37Rv RIF mono-resistant strain (Sharma *et al.*, 2010) and some MDR clinical isolates, but not in others (Siddiqi *et al.*, 2004; Gupta *et al.*, 2006; Jiang *et al.*, 2008; Louw *et al.*, 2011). The upregulation in *Rv1258c* expression in only

some strains indicates a possible effect of other SNPs present in the strains on *Rv1258c* expression.

A possible binding site for the global transcriptional regulator, WhiB7, has been identified in the promoter of *M. tuberculosis Rv1258c* (Burian *et al*., 2012). *Rv1258c* is regulated by WhiB7 and exposure to antibiotics initially increased *whiB7* expression followed by a *whiB7* dependent induction of *Rv1258c* (Morris *et al*., 2005).

Both *rpoB* mutations and efflux are thus important in the acquisition of RIF resistance and determining the levels of RIF resistance in *M. tuberculosis*. It is currently still unclear if there is a link between the *rpoB* mutation and expression of efflux pumps implicated in RIF resistance. This study will focus on expression of the *Rv1258c* and *whiB7* genes, in different *rpoB* mutants.

2.2 HYPOTHESIS, AIMS AND EXPERIMENTAL DESIGN

Overall aim:

To determine the effects of *rpoB* mutations on *Rv1258c* and *whiB7* expression in *M. tuberculosis*.

Hypothesis 1:

The presence of a *rpoB* mutation affects the expression of the *Rv1258c* multidrug efflux pump in *M. tuberculosis* H37Rv RIF mono-resistant mutants in the absence and presence of RIF.

Aim 1:

To monitor and compare *Rv1258c* expression in *in vitro* generated *M. tuberculosis* H37Rv RIF mono-resistant *rpoB* mutants in the absence and presence of RIF.

Experimental design:

The experimental design is outline in Fig 2.1. Briefly, promoter reporter vectors (episomal and integrating) with the *M. tuberculosis* H37Rv *Rv1258c* promoter upstream of a *lacZ* gene were constructed and functioning of these vectors tested in *M. smegmatis*. The effects of *rpoB* mutations on *Rv1258c* expression in *M. smegmatis* and *M. tuberculosis* were investigated

using β-galactosidase assays. The promoter activity results obtained during β-galactosidase assays were compared to the transcript levels of the native *Rv1258c* gene using qRT-PCR.

Hypothesis 2:

The presence of *rpoB* mutation affects the expression of the *whiB7* gene, encoding a transcriptional regulator of *Rv1258c*, in *M. tuberculosis* H37Rv RIF mono-resistant *rpoB* mutants.

Aim 2:

To monitor *whiB7* expression in *in vitro* generated *M. tuberculosis* RIF mono-resistant *rpoB* mutants in the absence of RIF using qRT-PCR.

Experimental design:

The experimental design is outline in Fig 2.1. Briefly, RNA was extracted and qRT-PCR used to determine transcript levels of *whiB7* in *M. tuberculosis rpoB* mutants.

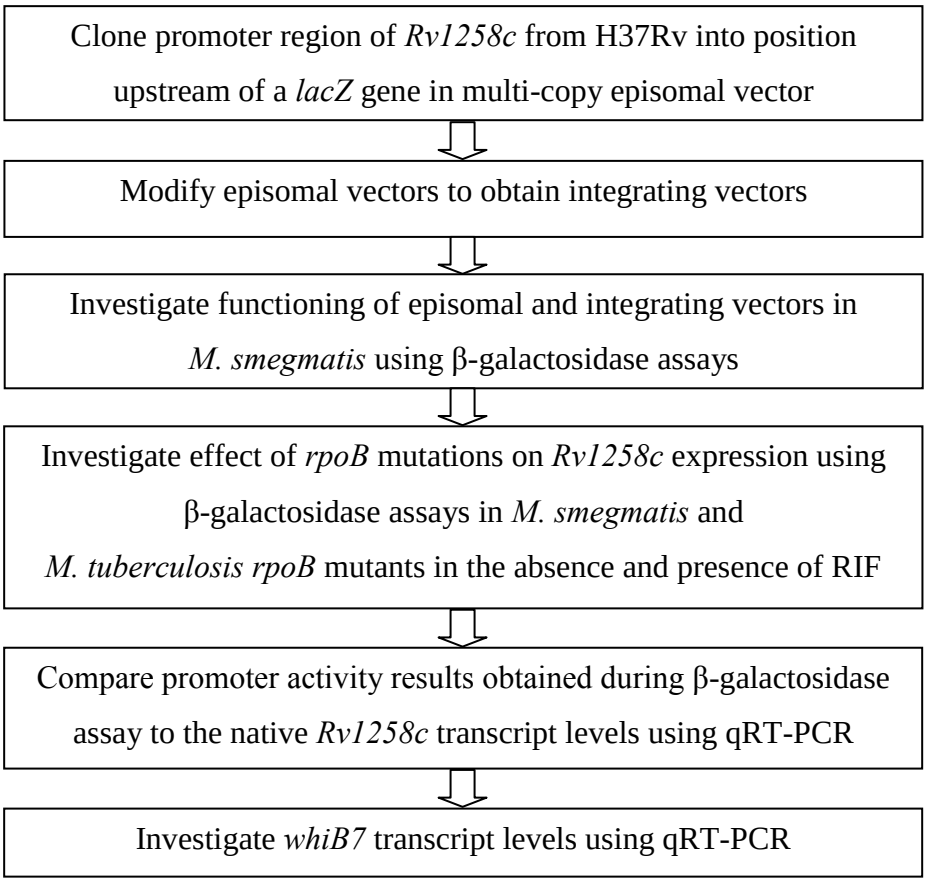


Figure 2.1. Experimental design of this study.

CHAPTER 3: GENERAL METHODOLOGY

3.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strains used during this study are summarized in Table 3.1. *E. coli* strain XL1Blue was used for all cloning purposes. *E. coli* was cultured overnight in Luria-Bertani (LB) broth, aerobically at 37°C with shaking at 175 rpm or on LB agar at 37°C. The following antibiotics were added to the LB broth and agar when necessary for selection: kanamycin (50 µg/ml), ampicillin (100 µg/ml) and hygromycin (150 µg/ml) (Sigma-Aldrich). When required 5-Bromo-4 Chloro-3 Indolyl β-D-galactosidase (X-gal) (40 µg/ml) (Roche Applied Sciences) (which was dissolved in dimethyl sulfoxide (DMSO)) was added to the LB agar.

M. smegmatis mc^2155 was cultured in Middlebrook 7H9 (Becton, Dickinson and Company) supplemented with 2% glucose, 0.85% sodium chloride and 0.05% Tween 80 (subsequently called 7H9 GS) aerobically at 37°C with shaking at 175 rpm or on 7H10 Difco Dubos Agar Base (Becton, Dickinson and Company) supplemented with 2% glucose and 0.85% sodium chloride (subsequently called 7H10 GS) at 37°C. *M. smegmatis* freezer stocks or single colonies were inoculated into a pre-culture, cultured to stationary phase and then sub-cultured to an optical density (OD) of 0.02 at 600 nm. The MIC for the *M. smegmatis* mc^2155 wild-type and *rpoB* mutant strains were previously determined and are summarized in Table 3.2.

M. tuberculosis was cultured under Biohazard safety three conditions in Middlebrook 7H9 (Becton, Dikinson and Company) supplemented with 0.2% glycerol, 0.05% Tween 80, albumin (0.5 µg/ml), 0.111% glucose and 0.0375% catalase (Sigma-Aldrich) subsequently called 7H9 ADC, unless stated otherwise. Culturing of *M. tuberculosis* on solid medium was done on Middlebrook 7H10 Agar Base (Becton, Dickinson and Company) supplemented with 0.5% glycerol, albumin (0.5 µg/ml), 0.111% glucose and 0.0375% catalase subsequently called 7H10 ADC, unless stated otherwise. Liquid culturing was done aerobically at 37°C in Greiner bio-one flasks without shaking in secondary airtight containers. *M. tuberculosis* freezer stocks or single colonies were inoculated into a pre-culture, cultured to an OD of approximately 1.00 at 600 nm and then sub-cultured to an OD of 0.05 at 600 nm.

The cultures were regularly screened for possible contamination by plating on blood agar that was checked for growth of contaminants two days after plating and Ziehl-Neelsen (Zn) staining. Zn-staining was done according to the protocol outlined by Kent & Kubica (1985). Briefly, a small drop of *M. tuberculosis* was heat fixed to glass slides using fixative and heating at 95°C for two hours. The slides were subsequently flooded with Carbol Fuschin, heated with a flame and left for 5 min. After rinsing with water, any non-acid fast bacteria (contaminants) were destained for 2 minutes (min) using acid-alcohol. After rinsing with water, counterstaining was done for 2 min with Methylene blue. Any non-acid fast bacteria would stain blue. The slides were rinsed and air dried. The slides were inspected using a light microscope under oil immersion lens for any possible contaminants.

The following antibiotics were added to the medium when necessary: kanamycin (50 µg/ml) and hygromycin (50 µg/ml) for *M. smegmatis* and *M. tuberculosis* and RIF, 10 µg/ml for *M. smegmatis* and 2 µg/ml for *M. tuberculosis*. RIF (Sigma-Aldrich) was dissolved in 10% DMSO (Sigma-Aldrich) to a final concentration of 1 000 µg/ml and was stored in aliquots at -80°C for no longer than a month. X-gal was added to a final concentration of 40 µg/ml to 7H10 GS as required. The vectors and primers used in this study are summarized in Table 3.3 and Table 3.4 respectively.

3.2 RESTRICTION ENZYME DIGESTS

Restriction enzyme (RE) digests were done in a total volume of 20 µl. The amount of enzyme added and incubation time was adjusted appropriately to ensure complete digestion of the DNA present in each reaction. Reaction mixes were incubated at 37°C. The amount of DNA digested varied and is specified for each digest in the figure titles.

3.3 AGAROSE GEL ELECTROPHORESIS

DNA fragments generated by restriction enzyme digests or PCR were separated by agarose gel electrophoresis using a Tris-EDTA buffer system (TAE) (containing Tris buffer (40 mM), Na_2EDTA (1 mM) and 0.112% glycerol). Electrophoresis was done at 100 V and an appropriate DNA ladder was included for sizing of the bands. The DNA was stained using ethidium bromide and visualized using an ultraviolet transilluminator.

3.4 LIGATIONS

For purification of DNA fragments prior to ligation, digested DNA or PCR products were separated by electrophoresis and the appropriate bands excised from the gel. The DNA was then purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.

Different ligation kits were used during this study as specified in the subsequent sections. The FastLink DNA Ligation kit (Epicentre Biotechnologies) was used according to the manufacturer's instructions. Briefly, 50 ng vector was added to the insert in a vector:insert ratio of 1:5 in a 15 µl ligation mixture containing 1x Fast-Link Ligation Buffer, 1 mM ATP and two units (U) Fast-Link DNA Ligase. After incubated for 15 min at room temperature, the ligase was inactivated by incubation at 65°C for 15 min.

3.5 PREPARATION OF ELECTROCOMPETENT *E. COLI*

E. coli was cultured to mid-log growth phase and chilled on ice for 20 min. The bacteria were harvested by centrifugation at 3,220 ×g for 15 min at 4°C, the supernatant discarded and bacteria washed three times with decreasing volumes of ice-cold 10% glycerol (equal volume to culture, followed by half of initial culture volume and $1/20^{th}$ of the initial culture volume). The bacterial pellet was re-suspended in $1/500^{th}$ the initial culture volume of 10% glycerol, snap frozen and stored at -80°C until required.

3.6 ELECTROPORATION OF *E. COLI*

Electrocompetent cells were thawed on ice. For each transformation, a 40 µl aliquot of electrocompetent cells was mixed with 2 µl of the ligation reaction or vector DNA and transferred into a Gene Pulser cuvette (Bio-Rad). Electroporation was done using a Bio-Rad MicroPulser (Bio-Rad) set to the EC2 setting for *E. coli* transformations. After electroporation, 500 µl LB broth was added and the bacteria were incubated at 37°C for one hour to allow expression of the selection marker, before plating on LB agar containing an appropriate antibiotic. Plates were then incubated as described above.

Table 3.1. Bacterial strains used

Bacterial strain	Vector	Description of strain	Provided by
E. coli XL1Blue	-	-	Laboratory collection
M. smegmatis mc^{2}155 wild-type	-	*ept-1* (efficient plasmid transformation) mutant of mc^{2}6	(Snapper *et al.*, 1990)
M. smegmatis mc^{2}155 *rpoB* S531L	-	Derivative of wild-type generated by allelic exchange containing TCG531TTG mutation at codon 531 of *rpoB* gene causing S531L amino acid substitution	Anastasia Koch at UCT (unpublished)
M. smegmatis mc^{2}155 *rpoB* H526Y	-	Derivative of wild-type generated by allelic exchange containing CAC526TAC mutation at codon 526 of *rpoB* gene causing H526Y amino acid substitution	Anastasia Koch at UCT (unpublished)
M. smegmatis mc^{2}155 wild-type (pJEM15)	pJEM15	*M. smegmatis* mc^{2}155 wild-type transformed with pJEM15 vector	This study
M. smegmatis mc^{2}155 wild-type (pJEM15promo)	pJEM15promo	*M. smegmatis* mc^{2}155 wild-type transformed with pJEM15promo vector	This study
M. smegmatis mc^{2}155 *rpoB* S531L (pJEM15)	pJEM15	*M. smegmatis* mc^{2}155 *rpoB* S531L transformed with pJEM15 vector	This study
M. smegmatis mc^{2}155 *rpoB* S531L (pJEM15promo)	pJEM15promo	*M. smegmatis* mc^{2}155 *rpoB* S531L transformed with pJEM15promo vector	This study
M. smegmatis mc^{2}155 *rpoB* H526Y (pJEM15)	pJEM15	*M. smegmatis* mc^{2}155 *rpoB* H526Y transformed with pJEM15 vector	This study
M. smegmatis mc^{2}155 *rpoB* H526Y (pJEM15promo)	pJEM15promo	*M. smegmatis* mc^{2}155 *rpoB* H526Y transformed with pJEM15promo vector	This study

Continued overleaf

Table 3.1. Bacterial strains used (continued)

Bacterial strain	Vector	Description of strain	Provided by
M. smegmatis mc^{2}155 wild-type *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. smegmatis* mc^{2}155 wild-type with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. smegmatis mc^{2}155 wild-type *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. smegmatis* mc^{2}155 wild-type with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. smegmatis mc^{2}155 *rpoB* S531L *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. smegmatis* mc^{2}155 *rpoB* S531L with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. smegmatis mc^{2}155 *rpoB* S531L *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. smegmatis* mc^{2}155 *rpoB* S531L with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. smegmatis mc^{2}155 *rpoB* H526Y *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. smegmatis* mc^{2}155 *rpoB* H526Y with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. smegmatis mc^{2}155 *rpoB* H526Y *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. smegmatis* mc^{2}155 *rpoB* H526Y with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv wild-type	-	H37RvJo (ATCC: 25618) (For comparison of genome with that of other H37Rv laboratory strains see Ioerger *et al.* (2010))	(Ioerger *et al*., 2010)
M. tuberculosis H37Rv rpoB S531L	-	Spontaneous *in vitro rpoB* TCG531TTG mutant causing S531L amino acid substitution generated from *M. tuberculosis* H37Rv wild-type strain and isolated by selection on RIF	This study
M. tuberculosis H37Rv rpoB H526Y	-	Spontaneous *in vitro rpoB* CAC526TAC mutant causing S531L amino acid substitution generated from *M. tuberculosis* H37Rv wild-type strain and isolated by selection on RIF	This study

Continued overleaf

Table 3.1. Bacterial strains used (continued)

Bacterial strain	Vector	Description of strain	Provided by
M. tuberculosis H37Rv wild-type (pJEM15promo)	pJEM15promo	*M. tuberculosis* H37Rv wild-type transformed with pJEM15promo vector	This study
M. tuberculosis H37Rv wild-type *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. tuberculosis* H37Rv wild-type with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv wild-type *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. tuberculosis* H37Rv wild-type with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv *rpoB* S531L *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. tuberculosis* H37Rv *rpoB* S531L with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv *rpoB* S531L *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. tuberculosis* H37Rv *rpoB* S531L with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv *rpoB* H526Y *attB*::pMV306*lacZ*	pMV306*lacZ*	*M. tuberculosis* H37Rv *rpoB* H526Y with pMV306*lacZ* vector integrated into *attB* site of mycobacterial genome	This study
M. tuberculosis H37Rv rpoB H526Y *attB*::pMV306promo*lacZ*	pMV306promo*lacZ*	*M. tuberculosis* H37Rv *rpoB* H526Y with pMV306promo*lacZ* vector integrated into *attB* site of mycobacterial genome	This study

Table 3.2. MICs of *M. smegmatis* mc^{2}155 wild-type and *rpoB* mutants

Bacterial strain	MIC for RIF	Method for determining MIC	Determined by
M. smegmatis mc^{2}155 wild-type	2.0 - 3.9 µg/ml	Microdilution assay in 7H9 supplemented with 10% oleic acid-dextrose-catalase (OADC)	A. Koch at UCT
M. smegmatis mc^{2}155 *rpoB* S531L	125 - 250 µg/ml	Microdilution assay in 7H9 supplemented with 10% OADC	A. Koch at UCT
M. smegmatis mc^{2}155 *rpoB* H526Y	125 - 250 µg/ml	Microdilution assay in 7H9 supplemented with 10% OADC	A. Koch at UCT

3.7 PREPARATION OF ELECTROCOMPETENT *M. SMEGMATIS*

M. smegmatis was cultured to mid-log phase, and then harvested by centrifugation at 4°C at 3,220 ×g. The cells were washed four times with decreasing volumes of ice-cold 10% glycerol. The bacterial pellet was finally re-suspended in 1/60th of the original volume of cold 10% glycerol. Electrocompetent *M. smegmatis* was incubated on ice until used.

3.8 PREPARATION OF ELECTROCOMPETENT *M. TUBERCULOSIS*

M. tuberculosis was cultured to an OD of approximately 1.00 at 600 nm and then harvested by centrifugation at 25°C at 3,220 ×g. The cells were washed two times with decreasing volumes of a mixture of 0.05% Tween 80 and 10% glycerol preheated to 37°C. The bacterial pellet was finally re-suspended in the volume needed for the amount of electroporations to be done (400 µl per electroporation) of the mixture of 0.05% Tween 80 and 10% glycerol. Electrocompetent *M. tuberculosis* was incubated at 37°C before use.

3.9 ELECTROPORATION OF *M. SMEGMATIS* AND *M. TUBERCULOSIS*

Electroporations were done at 37°C for *M. tuberculosis* and 4°C for *M. smegmatis* by either preheating or precooling the cells and cuvettes. A 400 µl aliquot of electrocompetent bacteria was mixed with the vector DNA (500 ng) and transferred to a Gene Pulser cuvette (Bio-Rad). The vectors used in this study are summarised in Table 3.3. Electroporation was done using a Bio-Rad Pulse Controller Plus using the following parameters: 1,000 Ω, 25 µF and 2,500 V. After electroporation, 800 µl of liquid media (7H9 GS or 7H9 ADC, for *M. smegmatis* or *M. tuberculosis* respectively) was added and the bacteria were incubated at 37°C for three hours in the case of *M. smegmatis* or overnight in the case of *M. tuberculosis* to allow expression of the selection markers before plating on 7H10 GS or 7H10 ADC containing the appropriate antibiotic. Transformants were selected.

3.10 COLONY PCR

Colony PCR was used to confirm the presence of the *Rv1258c* promoter region and or *lacZ* gene in the constructed vectors and to confirm that the bacteria have been successfully

transformed with the vectors. For *E. coli* and *M. smegmatis*, colonies were picked and resuspended in 10 µl nuclease free water. After spotting 2 µl of each suspension on appropriate solid media containing antibiotic to maintain the vector, the remaining suspension was heated to 100ºC for 5 min to facilitate cell lysis and DNA release. For *M. tuberculosis,* 10 ml 7H9 ADC was inoculated with the colony and the culture was allowed to grow for a few days. A 200 µl aliquot of this culture was transferred to an eppendorf tube and boiled for 30 min at 99°C to facilitate cell lysis and to ensure killing of the bacteria. The FastStart Taq DNA Polymerase kit (Roche Applied Sciences) was used to perform colony PCR. Each 12.5 µl PCR reaction mix contained 1× PCR buffer with magnesium chloride ($MgCl_{2)}$ (final concentration 2 mM $MgCl_2$), 1× GC-Rich solution, 200 µM of each deoxynucleoside triphosphate (dNTP) (Fermentas), 0.5 µM of both the forward and reverse primers, 1 µl cell lysates and 1.32 U FastStart Taq DNA Polymerase. The PCR cycling conditions were: initial denaturation at 95ºC for 4 min followed by 40 cycles of denaturation at 95ºC for 30 s, annealing at the appropriate annealing temperature (Tm) for the primers for 30 s and extension at 72ºC for 1 min followed by a final extension at 72ºC for 7 min. The primers used in this study are summarised in Table 3.4. Agarose gel electrophoresis was used to determine the size of the PCR products.

3.11 PLASMID DNA EXTRACTION

The NucleoBond PC100 Plasmid DNA Purification kit (Macherey-Nagel) was used according to the manufacturer's instructions for the isolation and purification of the following vectors: pJEM15, pJET1.2promo and pMV306promo*lacZ*. The Wizard Plus SV Minipreps DNA Purification System (Promega) was used according to manufacturer's instructions for the isolation and purification of the following vectors: pJEM15promo, pMV306, pMV306*lacZ* and pGEM-T EasyRACE1/2. All DNA concentrations and the quality of the DNA (260/280 ratios) was assessed using a NanoDrop ND-1000 spectrophotometer.

3.12 PREPARATION OF WHOLE CELL LYSATES (WCLS) FOR *M. SMEGMATIS* AND *M. TUBERCULOSIS*

WCLs were prepared from liquid cultures as previously described (Carroll & James, 2008). Briefly, *M. smegmatis* or *M. tuberculosis* culture (10 ml) was pelleted by centrifugation at 3,220 ×g for 10 min at 25°C. The pellet was washed with the original volume of 10 mM Tris

buffer (pH 8.0) (subsequently called Tris buffer), re-suspended in 1 ml Tris buffer and transferred to a 2 ml screw cap tube containing 0.1 mm glass beads (Biospec Inc). The bacteria were lysed in a Biospec Bead Beater at speed 6.00 for 30 s in the case of *M. smegmatis* and three times 25 s at speed 5.00 in the case of *M. tuberculosis*. The cellular debris was pelleted by centrifugation at 16,100 ×g for 10 min and the supernatant transferred to a sterile 1.5 ml eppendorf tube. For *M. smegmatis* WCLs, the supernatant was clarified by centrifugation at 16,100 ×g for 5 min and transferred to a sterile eppendorf tube. For *M. tuberculosis,* the WCLs were double filter sterilized using Acrodisc Syringe Filters (Pall Corporation). The WCLs were stored in two aliquots at -80°C until used for the Bradford and β-galactosidase assays.

3.13 PROTEIN CONCENTRATION DETERMINATION

The protein concentrations for the WCLs were determined using Bradford protein determination assays (Bradford, 1976). A stock solution containing 8 mg/ml of Bovine serum albumin (BSA) in Tris buffer was prepared and stored in aliquots at -20°C. Dilutions of the BSA stock were used to generate a standard curve (0.05 - 0.25 mg/ml) for each assay and Tris buffer was used as a blank. The assays were done in 96 well microtitre plates (Greiner Bio-one) in triplicate. Each assay contained 10 µl BSA dilution or WCL and 200 µl Bradford reagent (Bio-Rad), the latter pre-warmed to room temperature. The microtitre plate was covered with foil to prevent exposure of the reaction to light and incubated at room temperature for 15 min prior to measuring the absorbance at 595 nm using a Bio-Tek Synergy HT plate reader (Bio-Tek instruments) and the KCL4 program and Excel was used for analysis of the data. Appropriate dilutions of the WCLs were made to ensure the concentrations could be determined using the linear range of the standard curve. The protein concentration for each WCL was determined using the following formula:

$$\frac{\text{Average of three absorbance readings at 595 nm} - \text{Blank absorbance}}{\text{Gradient of protein trend line}} \times \text{Dilution factor}$$

3.14 β-GALACTOSIDASE ASSAYS

The WCLs were defrosted on ice and diluted in Tris buffer to the same total protein concentration. A twofold dilution series of each sample was then prepared in order to

determine the appropriate protein concentration to use in the assay for each strain. This is required since the amount of β-galactosidase in each sample may vary relative to the amount of total protein in the different strains. O-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma-Aldrich) was freshly prepared before each assay by dissolving ONPG in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$) to a concentration of 1.33 mg/ml and it was filter sterilized. The β-galactosidase assays were done in a microtitre plates in triplicate and the reaction was initiated by mixing 50 µl ONPG (final concentration 665 µg/ml) with 50 µl of the WCL dilutions. The microtitre plate was placed in a Bio-Tek Synergy HT plate reader (Bio-Tek instruments) within 5 min of adding the ONPG and the absorbance at 420 nm was measured every 5 min for 1 hour. The absorbance for the Tris buffer was used as blank and subtracted from each reading. The kinetic reads were plotted to generate kinetic curves at different protein concentrations of each of the dilutions of the WCLs. An appropriate protein concentration and incubation period was selected (refer to Addendum B for an explanation of how this was done) and used to calculate the β-galactosidase activity in Miller units according to the following formula:

$$\beta\text{– galactosidase activity (Miller units)} = \frac{\text{Average of three absorbance readings at 420 nm} - \text{absorbance of blank}}{\text{Final [Protein] in assay (mg/ml)} \times \text{Time exposed (min)} \times \text{Total volume of assay (ml)}}$$

3.15 RNA EXTRACTION

3.15.1 RNA extraction using trizol

The trizol method of RNA extraction was used as described previously in (Betts *et al.*, 2002) with slight modifications. Briefly, 10 ml of the culture was pelleted by centrifugation at 3,220 ×g and re-suspended in 1 ml trizol (Applied Biosystems, Ambion). The cell suspension was transferred to tubes containing 0.1 mm glass beads and ribolysed in a Biospec Bead Beater at speed 6.0 for two 60 s cycles with 60 s cooling intervals in between. After centrifugation the supernatant was transferred to a new tube and 120 µl chloroform added. Tubes were inverted rapidly for 15 s and periodically inverted for 2 min. After centrifugation at 14,000 ×g, the top layer was transferred to a new tube, 200 µl chloroform added and the tubes inverted as previously. An equal volume of isopropanol was added and samples were incubated for 10 min. The RNA was pelleted by centrifugation at 14,000 ×g and pellet

washed with 70% ethanol. Centrifugation at 14,000 ×g was used to pellet the RNA, the ethanol was removed and the pellet was air-dried. The RNA was re-suspended in 1x DNase I buffer (Applied Biosystems, Ambion) and incubated with 4 U of DNase I (Applied Biosystems, Ambion) for 1 hour. The RNeasy kit (Qiagen) was used for RNA clean-up according to the manufacturer's instructions. RNA samples, treated with 350 µl RLT buffer (from the RNeasy kit) and 250 µl ethanol, were loaded onto a column and spun down for 15s. In order to concentrate RNA, multiple preparations was done from a single culture and all the RNA loaded onto a single column. The RNA was eluted in 60 µl nuclease free water. RNA (49.2 µl) was added to 1× Turbo DNase buffer (Applied Biosystems, Ambion) and incubated with 9.6 U Turbo DNase at 37ºC for 2 hours. Phenol-chloroform extraction of the RNA was done and the RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer.

3.15.2 RNA extraction using FastRNA Pro Blue Kit

The FastRNA Pro Blue kit (Qbiogene) was used for RNA extractions according to the manufacturer's instructions. *M. tuberculosis* was cultured to the appropriate OD at 600 nm and 10 ml culture pelleted at 3,220 ×g. The supernatant was discarded, the pellet re-suspended in 1 ml RNApro solution and the re-suspended cells transferred to a 2 ml tube containing Lysing Matrix B. The samples were rybolysed five times for 25 s at speed 4.5 and the cellular debris pelleted at 12,000 ×g for 15 min at 4°C. The supernatant was transferred to an eppendorf tube, 300 µl chloroform added and vortexed for 10 s. After 5 min incubation at room temperature to facilitate nucleoprotein dissociation, the phases were separated by 10 min centrifugation at 12,000 ×g at 4°C. The upper phase was transferred to an eppendorf tube. Cold absolute ethanol (500 µl) was added and the samples were vortexed. The sample was stored for at least 1 hour at -20°C to precipitate the RNA. The RNA was pelleted by centrifugation at 12,000 ×g for 30 min at 4°C. Cold 75% ethanol (500 µl) was added to the pellet after removal of the supernatant and the sample was vortexed for 5 s. The ethanol was removed and the sample air dried for 5 min at room temperature. Water was added to the RNA pellet and the sample was incubated for minimum 5 min at room temperature to facilitate RNA re-suspension.

The samples were then treated with DNase I (8 U per 100 µl) in 1× DNase I buffer (Applied Biosystems, Ambion) for 45 min at 37°C, unless stated otherwise. The RNA was precipitated

overnight at 4°C as described below. After the RNA pellet was re-suspended in water, the RNA was incubated with Turbo DNase I (2 U) in 1× Turbo DNase buffer (Applied Biosystems, Ambion) at 37°C for 30 min. The same amount of U of Turbo DNase I (2 U) was added after 30 min of incubation and the sample was incubated for a further 30 min at 37°C. The enzyme was inactivated by phenol-chloroform extraction and the RNA precipitated overnight as described below. After re-suspending the RNA in water, the RNA quality (the 260/280 ratio) was assessed and the concentration was determined using a NanoDrop ND-1000 spectrophotometer. PCR was done to determine whether the RNA was free of genomic DNA as described below. It is important to note that the amount of U of DNase I and Turbo DNase I used for the removal of the genomic DNA from the RNA samples differed in some instances according to the concentration of the crude RNA extraction as determined using the NanoDrop ND-1000 spectrophotometer after the first steps of the extraction.

3.15.3 Precipitation of RNA

The volume of the RNA sample was made up with water to a total of 200 µl. An equal volume of phenol-chloroform (a 4:1 ratio mixture) was added, vortexed and placed on ice for 10 min, vortexing every 3 min. After centrifugation at room temperature for 10 min at 13,000 ×g, to separate the layers, the top layer (150 µl) was transferred to an eppendorf tube. This was done to inactivate the DNase I and to remove it from the sample. Sodium acetate (0.1× volume) and 100% ethanol (2.5× volume) was added and the sample vortexed. The sample was incubated overnight at 4°C, spun down at 13,000 ×g for 30 min at 4°C and the pellet washed with 75% ethanol. After centrifugation for at 10,000 ×g for 2 min at room temperature, the ethanol was removed and the pellet air dried for 5 min. Water was added and the sample incubated at room temperature for at least 5 min to facilitate RNA re-suspension.

3.15.4 PCR to check whether the RNA samples were clean of genomic DNA

PCR was used to assess whether the RNA sample contained any genomic DNA. Each 12.5 µl PCR reaction mix contained 1× PCR buffer with $MgCl_2$ (final concentration of 2 mM), 1× GC-Rich solution, 200 µM dNTPs, 0.5 µM *Rv1258c*pF and *Rv1258c*pR primers, 1 µl RNA sample and 1.32 U FastStart Taq DNA polymerase (Roche Applied Sciences). The primers used are summarised in Table 3.4. Genomic DNA of *M. tuberculosis* H37Rv was also

amplified as positive control. The PCR cycling conditions were: initial denaturation at 95ºC for 4 min followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 60ºC for 30 s and extension at 72ºC for 1 min and a final extension at 72ºC for 7 min. The PCR products were analysed using agarose gel electrophoresis.

3.16 STATISTICAL ANALYSES

Unpaired t-tests were used to analyse the significance of differences between datasets and were performed in GraphPad Prism 5. The Welch correction was used whenever variance within samples was not the same for the samples being compared, in order to correct for variance within samples.

Table 3.3. Vectors used in this study

Vector	Episomal/ Integrating	Copy number	Size (bp)	Description of vector	Originals obtained from
pJET1.2	Episomal	High (in *E. coli*)	2,974	Contains: • a β-lactamase gene, *bla* (ApR), conferring ampicillin resistance • a lethal gene, *Eco*47IR, encoding toxic restriction endonuclease Eco47I, modified to contain a multiple cloning site (MCS) • The vector is linearized at position 371 providing blunt DNA ends for the ligation of an insert	CloneJET PCR Cloning Kit (Fermentas)
pJET1.2 promo	Episomal	High (in *E. coli*)	3,621	pJET1.2 vector containing the 635 bp promoter region upstream of the *M. tuberculosis Rv1258c* gene (amplified by PCR) cloned into the MCS at position where linearized	This study
pJEM15	Episomal	High (over 500 copies in *E. coli* and 3-10 copies in mycobacteria)	9,446	An *E. coli*-mycobacteria shuttle vector containing: • aminoglycoside phosphotransferase gene (*aph*), which confers kanamycin resistance • a reporter cassette with a mycobacterial transcriptional terminator followed by a MCS allowing promoter and operon fusion to the reporter gene • β-galactosidase gene *(lacZ)* devoid of its regulatory sequences • an *E. coli* replicon allowing preparation and manipulation of vector in *E. coli* • a mycobacterial replicon allowing expression of the vector in mycobacteria	(Timm *et al.*, 1994)

Continued overleaf

Table 3.3. Vectors used in this study (continued)

Vector	Episomal/ Integrating	Copy number	Size (bp)	Description of vector	Originals obtained from
pJEM15 promo	Episomal	High (3-10 copies in mycobacteria and over 500 copies in *E. coli*)	10,075	pJEM15 vector containing the 635 bp promoter region upstream of the *M. tuberculosis Rv1258c* gene (from pJET1.2promo) cloned into the MCS upstream of the *lacZ* gene	This study
pMV306	Integrating	Single copy	4,271	Contains: • a hygromycin resistance gene • an integrase gene which allows the vector to integrate into the *attB* site of the mycobacterial genome	(Stover *et al.*, 1991)
pMV306 *lacZ*	Integrating	Single copy	7,536	pMV306 vector with the *lacZ* gene and the MCS of the pJEM15 vector and the transcriptional terminator upstream of that, cloned into it	This study
pMV306 promo*lacZ*	Integrating	Single copy	8,165	pMV306 vector with the promoter region of the *M. tuberculosis* H37Rv *Rv1258c* gene fused to the *lacZ* gene and transcriptional terminator upstream of the MCS from the pJEM15promo vector cloned into it	This study
pGEM-T Easy	Episomal	High	3,015	Contains: • an ampicillin resistance gene, amp^R • a MCS within the alpha-peptide coding region of the *lacZ* gene encoding β-galactosidase • T7 and SP6 RNA polymerase promoters flanking the MCS	Promega

Table 3.4. Primers used in this study

Primer	**Sequence 5' – 3'**	**Tm °C**
Initial amplification of *Rv1258c* promoter region		
pRv1258cpF	GGT ACC GAA TAT CGC GGC TGA ATC TA	60.0
pRv1258cpR	GGA TCC CAT TTA GCC AGG GTG AGC AT	62.5
Sequencing of *Rv1258c* promoter region in sequencing vector pJET1.2		
pJET1.2For	CGA CTC ACT ATA GGG AGA GCG GC	NA
pJET1.2Rev	AAG AAC ATC GAT TTT CCA TGG CAG	NA
Used during PCR to check whether transformation of bacteria with the vector systems was successful		
*LacZ*For	CTG GCG TAA TAG CGA AGA GG	55.3
*LacZ*Rev	GTT GCA CCA CAG ATG AAA CG	54.7
Used for the amplification and subsequent sequencing of the *rpoB* RRDR		
rpoB For	TGG TCC GCT TGC ACG AGG GTC AGA	66.3
rpoB Rev	CTC AGG GGT TTC GAT CGG GCA CAT	63.1
5'Rapid amplification of cDNA ends (5' RACE) primers		
Oligo d(T)-anchor	GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV V=A, C or G	NA
PCR anchor	GAC CAC GCG TAT CGA TGT CGA C	59.8
Control primer neo1/rev	CAG GCA TCG CCA TGG GTC AC	61.4
Control primer neo2/rev	GCT GCC TCG TCC TGC AGT TC	61.1
Control primer neo3/rev	GAT TGC ACG CAG GTT CTC CG	59.0
RACE1 SP1	CAA TAA AGG CCA GGT TGA GGA TCG CC	61.6
RACE1 SP2	ACT ATC GAG ACG CCA TCA CCC G	60.8
RACE2 SP1	AGC TGG CGA AAG GGG GAT GT	61.8
RACE2 SP2	AAC GCC AGG GTT TTC CCA GTC	60.3
RACE2 SP3	GGG TTT TCC CAG TCA CGA CGT T	59.8
pGEMTeasyT7	TAA TAC GAC TCA CTA TAG GG	NA
pGEMTeasySP6	TAT TTA GGT GAC ACT ATA G	NA
qRT-PCR primers		
MTB-SigA-RT	CTG ACA TGG GGG CCC GCT ACG TTG	66.0
Rv1258c-RT	TGT TGG CTA TCC GCG TTC	55.5
whiB7-RT	GAT CTG TGG TTC GCC GAT AC	55.5
MTB-SigA-F1	TGC AGT CGG TGC TGG ACA C	60.5
MTB-SigA-R1	CGC GCA GGA CCT GTG AGC GG	66.0
Rv1258c-F1	CCC GCG TCT GTA TCA CGT A	56.7
Rv1258c-R1	CGG TTC TTA CCC TGG GTT TG	55.8
whiB7-F1	TCG AGC CTT GGT CGA ATA TC	54.2
whiB7-R1	CTC GAG GTA GCC AAG ACA CTG	57.1

CHAPTER 4: VECTOR CONSTRUCTION

4.1 INTRODUCTION

The expression of mycobacterial genes in other bacterial systems was initially limited because of the inability of regulatory elements in other bacteria to recognise and subsequently express mycobacterial genes. This is as a result of the evolutionary distance of the bacterial species from mycobacteria (Machowski *et al.*, 2005). In 1985 mycobacterial DNA was expressed for the first time in the model organism *E. coli*. This opened up new possibilities for studying mycobacterial gene expression using molecular genetics tools such as protein expressing genes and episomal and integrating vectors. Subsequently, shuttle vectors that are able to replicate in mycobacteria and can be used to transform mycobacteria were developed, creating new research possibilities (Machowski *et al.*, 2005).

Promoter activity assays are frequently used to study gene expression and involve the creation of transcriptional fusion of a heterologous promoter to a promoterless reporter gene upstream of the gene, allowing for investigating transcriptional regulation upon exposure to different environmental conditions (Machowski *et al.*, 2005; Carrol & James, 2008). Reporter genes that have been used in mycobacteria include *gfp*, encoding green fluorescent protein, and *lacZ*, encoding β-galactosidase. The *lacZ* gene can be used to quantitatively determine the promoter activity from WCLs in liquid assays (Machowski *et al.*, 2005; Carrol & James, 2008).

β-galactosidase is an enzyme that cleaves the colourless substrate ONPG to a yellow chromophore with a maximum absorbance at 420 nm. The absorbance increase at 420 nm over the assay period is therefore directly proportional to the β-galactosidase concentration and the promoter activity. The *lacZ* reporter is advantageous since the β-galactosidase protein is stable and no background β-galactosidase activity is present in mycobacteria. The β-galactosidase assays also enable the study of promoters with low *in vitro* activity because of its sensitivity (Carrol & James, 2008).

The pJEM series of *E. coli*-mycobacteria promoter reporter shuttle vectors were designed by Timm *et al.* (1994). In these vectors, an *E. coli* and mycobacterial replicon are present to ensure replication in both bacterial systems. A *lacZ* gene, devoid of any regulatory sequences,

serves as the reporter gene. A heterologous sequence or promoter can be fused to the amino terminus of the *lacZ* gene without loss of enzymatic activity. The pJEM15 vector contains a transcriptional terminator upstream of the MCS. The selectable marker gene, *aph*, confers kanamycin resistance and kanamycin is thus added to the media in order to select for the presence of the vector. This episomal vector is a multi-copy vector in *E. coli* and mycobacteria (Timm *et al.*, 1994).

In order to investigate *M. tuberculosis* H37Rv *Rv1258c* promoter activity in this study, the promoter region of *Rv1258c* would be cloned into the pJEM15 vector upstream of the *lacZ* gene. Since the pJEM15 vector is a multi-copy vector system, we hypothesized that the regulation of *Rv1258c* promoter could be altered by the multiple copies of the *Rv1258c* promoter present within the cell. In addition, the presence of the selectable drug (kanamycin) in the media could also influence the expression of the *Rv1258c* promoter. We therefore sought to improve the pJEM15 reporter system.

The discovery of the gene responsible for the integrating of lysogenic mycobacteriophages into the mycobacterial genome has led to the development of integrating vectors. Integrating vectors have an *int* gene encoding integrase, which permits integrating of phage DNA into the mycobacterial DNA. An *attP* site (from a phage) is also present in the integrating vectors and recombination of the *attP* site with the chromosomal *attB* site in the mycobacterial genome leads to integration of the entire vector into the *attB* site of the chromosome (Machowski *et al.*, 2005). Only one copy of the integrating vector can integrate into the mycobacterial genome. Integrating vectors must integrate into the mycobacterial genome in order to persist since they lack a mycobacterial origin of replication (Dennehy & Williamson, 2005). Integrating vectors are generally more stably maintained in the mycobacterial genome than episomal vectors and less likely to mutate once integrated, probably because only a single copy of the vector is present per cell (Dennehy & Williamson, 2005). It may therefore be possible to culture transformants in the absence of the selectable drug. The pJEM15 vector was therefore modified to integrate into the *attB* site of the mycobacterial genome. The generation of episomal and integrating versions of the reporter vectors containing the *M. tuberculosis Rv1258c* promoter region would allow the validation of this new system.

4.2 METHODOLOGY

4.2.1 Determining the transcriptional start site of *Rv1258c*

4.2.1.1 5' RACE

In order to determine the position of the transcriptional start site of the *M. tuberculosis Rv1258c* gene, 5' RACE was done. The principle of 5' RACE is summarized in Fig 4.1. Briefly, this involves synthesis of cDNA using a gene-specific primer, polyA-tailing of the cDNA by recombinant terminal transferase and amplification of the cDNA using an oligo d(T)-anchor primer and nested gene specific primer.

The 5'/3' RACE Kit 2nd Generation (Roche Applied Sciences) was used according to the manufacturer's instructions. This kit contains a control system in the form of a control neo-RNA (an *in vitro* transcribed RNA) and three control RNA specific neo-primers which allow for testing whether the cDNA synthesis, purification, polyA-tailing and the PCR amplification steps are working. The neo-RNA and gene specific control neo-primers are added to the RNA sample in the control reaction, but are omitted in the experiment (which only contains the RNA of interest). The primers used during 5' RACE are summarised in Table 3.4.

First-strand cDNA synthesis was done in a total volume of 20 µl. Each reaction contained 1× cDNA synthesis buffer, 1 mM dNTPs, 0.625 µM cDNA synthesis reverse transcription primer (SP1), the RNA sample and 25 U Transcriptor Reverse Transcriptase. The cDNA was subsequently purified using the High Pure PCR Product Purification Kit (Roche Applied Sciences) according to the modified protocol provided within the 5'/3' RACE Kit 2nd Generation kit. A homopolymeric A-tail was added to the 3' end of the first-strand cDNA using recombinant terminal transferase. Each 25 µl reaction contained 1× Reaction Buffer, 200 µM dATP, 80 U Terminal Transferase and 19 µl purified cDNA.

PCR amplification of the polyA-tailed cDNA was done in a 50 µl reaction containing 1× PCR Reaction Buffer (supplied with Taq DNA Polymerase, Roche Applied Sciences), 200 µM dNTPs, 0.75 µM oligo dT-anchor primer, 0.25 µM of a second nested gene specific primer (SP2), 2.5 U Taq DNA Polymerase and 5 µl polyA-tailed cDNA.

The PCR cycling conditions were: initial denaturation at 95ºC for 4 min, 10 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s, 25 cycles of denaturation at 95ºC for 30 s annealing at 55ºC for 30 s and extension at 72ºC with an increase of 20 s in extension time with every cycle and a final extension at 72ºC for 7 min. The PCR products (10 µl of each) were analysed using agarose gel electrophoresis.

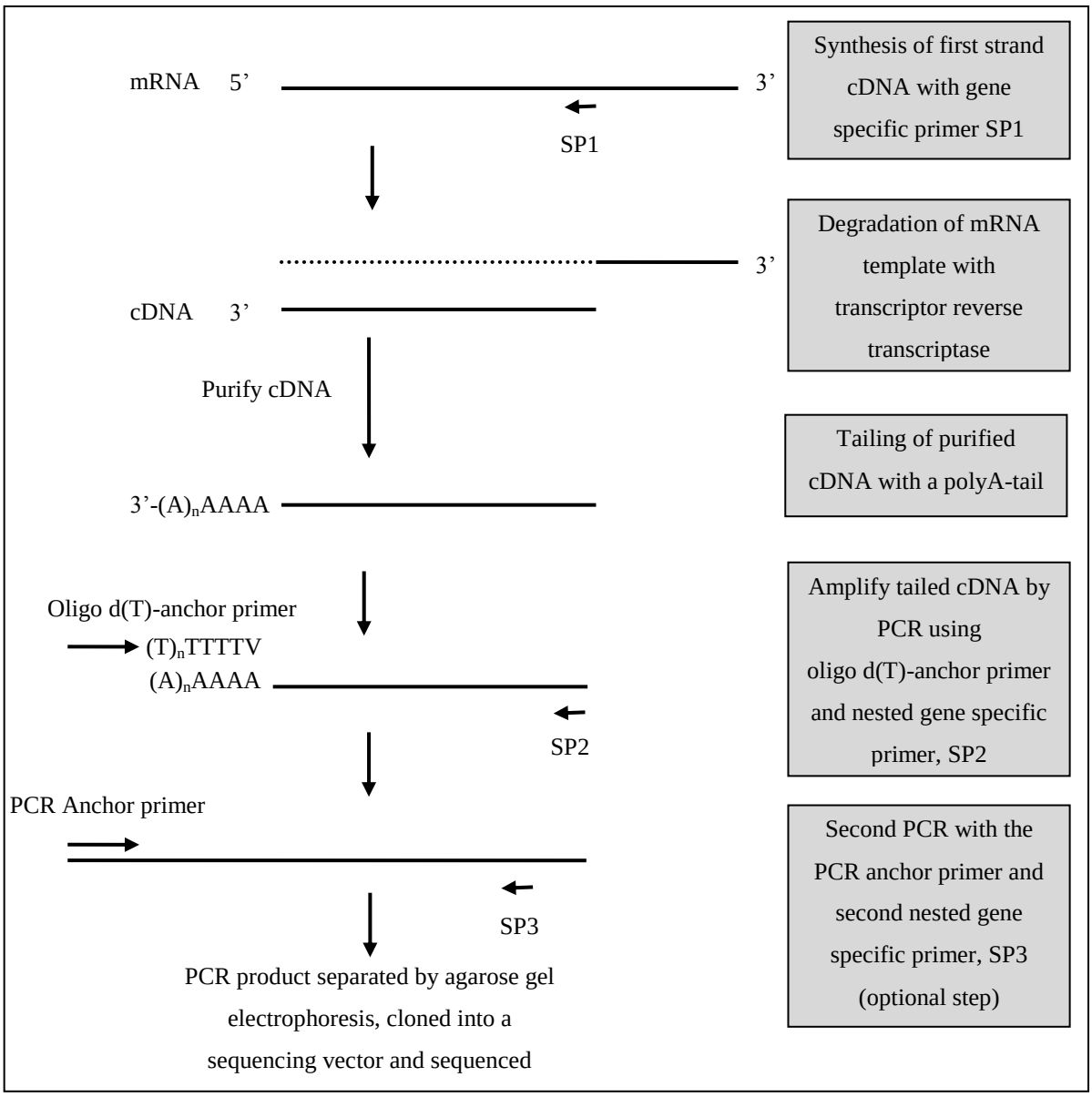


Figure 4.1: Principle of 5' RACE as summarised in the 5'/3' RACE 2nd Generation kit.

4.2.1.2 Control reactions of 5' RACE

For the control reactions the neo-RNA and gene specific control neo-primers are added to the RNA sample in the control reaction before the reverse transcription step. The cDNA control sample contained all the reagents previously described for reverse transcription reaction, but

contained an additional 1 ng control RNA and 0.625 µM neo1/rev primer (which bind specifically within the control RNA). The cDNA control reaction was also purified and a homopolymeric A-tail was added to the 3' end of the first-strand control cDNA using recombinant terminal transferase as described for the cDNA sample.

PCR amplification of the unpurified and purified control cDNA was done in order to determine whether the reverse transcription step was successful. The expected size of the product for the control cDNA was 157 bp. Each 50 µl reaction contained 1× PCR Reaction Buffer (supplied with the Taq DNA Polymerase, Roche Applied Sciences), 200 µM dNTPs, 0.25 µM Control primer neo2 and Control primer neo 3, 2.5 U Taq DNA Polymerase and 1 µl control cDNA. As an extra check, PCR amplification of the unpurified and purified cDNA was done. No product was expected for the PCR containing the cDNA sample, as no control RNA was present in this sample.

PCR was also done, as a control, to determine whether the polyA-tailing step was successful. A product of 293 bp was expected for the polyA-tailed control cDNA. Each 50 µl reaction contained 1× PCR Reaction Buffer (supplied with Taq DNA Polymerase, Roche Applied Sciences), 200 µM dNTPs, 0.75 µM oligo d(T)-anchor primer, 0.25 µM Control primer neo 2, 2.5 U Taq DNA Polymerase and 1 µl control cDNA.

4.2.1.3 Cloning of 5' RACE product into pGEM-T Easy vector

The RACE PCR products were either purified directly or first separated by electrophoresis and then purified from the gel before cloning into pGEM-T Easy. *E. coli* was transformed with the ligation mixture and cultured on LB agar containing ampicillin. White colonies were selected and colony PCR was done using the PCR anchor primer (supplied with the 5'/3' RACE 2nd Generation kit - Roche Applied Sciences), and nested gene specific primers (SP2/SP3). The Central Analytical Facility at Stellenbosch University sequenced the insert using the pGEMTeasyT7 and/ or pGEMTeasySP6 primer.

4.2.2 Cloning *Rv1258c* promoter region into pJET1.2

A 635 bp region directly upstream of the *Rv1258c* gene of *M. tuberculosis* H37Rv was amplified by PCR using the pRv1258cpF and pRv1258cpR primers which contained RE

cutting sites, *Asp*718 and *Bam*HI respectively (Fig 4.2). This region presumably contained the transcriptional start site of *Rv1258c* and will subsequently be called the promoter region. Each 50 µl PCR reaction mix contained 1× Phusion HF Buffer, 1× GC Buffer (from the FastStart Taq DNA polymerase kit) (Roche Applied Sciences), 200 µM of each dNTP (Fermentas), 0.5 µM pRv1258cpF and pRv1258cpR, 200 ng mycobacterial DNA and 1 U Phusion Hot Start DNA Polymerase (Thermo Fisher Scientific, Finnzymes). The PCR cycling conditions were as follows: Initial denaturation at 98ºC for 1 min followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 60ºC for 30 s and extension at 72ºC for 30 s and a final extension at 72ºC for 7 min.

The PCR product was purified and ligated into the pJET1.2 vector (Fig 4.2) using the FastLink DNA Ligation kit (Epicentre Biotechnologies). After transformation of *E. coli* with the ligation reaction, selection of the transformants was done on LB agar containing ampicillin. Colonies containing an insert were inoculated into 5 ml LB broth. Vector DNA was extracted using the plasmid DNA extraction kits previously described and the insert sequenced using the pJET1.2For and pJET1.2Rev primers to confirm that no mutations were introduced during PCR. The resulting vector will subsequently be called pJET1.2promo.

4.2.3 Cloning of *Rv1258c* promoter region into pJEM15

The promoter region of *Rv1258c* was subsequently fused to *lacZ* in pJEM15 in order to create a promoter reporter construct (Fig 4.3). pJEM15 and pJET1.2promo were digested with *Asp*718 and *Bam*HI and the DNA fragments were separated by agarose gel electrophoresis The appropriate bands were excised and purified. pJEM15 was dephosphorylated using Antarctic phosphatase (Biolabs) according to the manufacturer's instructions and ligation was carried out using the T4 DNA Ligase kit (Promega) overnight at 4°C according to the manufacturer's instructions. After transformation of *E. coli* with the ligation reaction, transformants were selected on LB agar containing kanamycin. Colony PCR was done to confirm the presence of the promoter region. The resulting vector will subsequently be called pJEM15promo.

4.2.4 Construction of integrating vectors

The promoterless *lacZ* gene from pJEM15 and promoter fused to *lacZ* gene of pJEM15promo

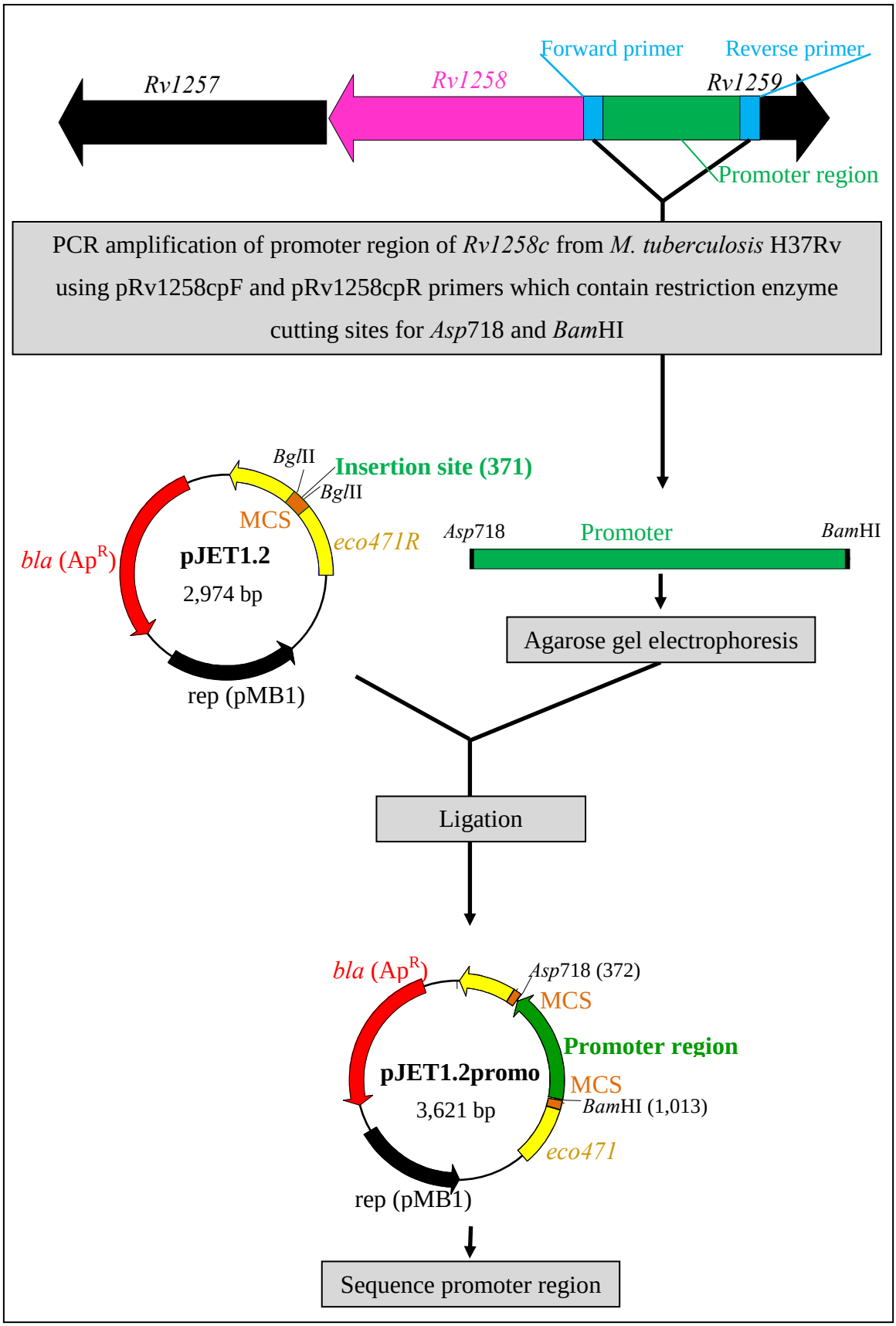


Figure 4.2. Amplification of the promoter region of *Rv1258c* from *M. tuberculosis* H37Rv and cloning into pJET1.2.

were subcloned into the integrating vector pMV306 as follows: pMV306 was digested with *Eco*RV, gel purified and dephosphorylated using rAPID Alkaline Phosphatase (Roche Applied Sciences), according to the manufacturer's instructions. JEM15 and pJEM15promo were digested with *Pst*I and *Pst*I/*Eco*RI respectively, and the bands containing the *lacZ* gene were gel purified. Blunting of the DNA fragments from the pJEM15 (228.88 ng) and pJEM15promo (113.19 ng) vectors were done using the DNA blunting enzyme and 1× reaction buffer from the CloneJET PCR Cloning Kit (Fermentas) according to the manufacturer's instructions. The dephosphorylated vector (50 ng) was added to the blunting enzyme mixture and Fast-Link DNA Ligase was used for ligation overnight at 4°C (Fig 4.4 and Fig 4.5). After transformation of *E. coli* with the ligation reactions, transformants were selected on LB agar containing hygromycin.

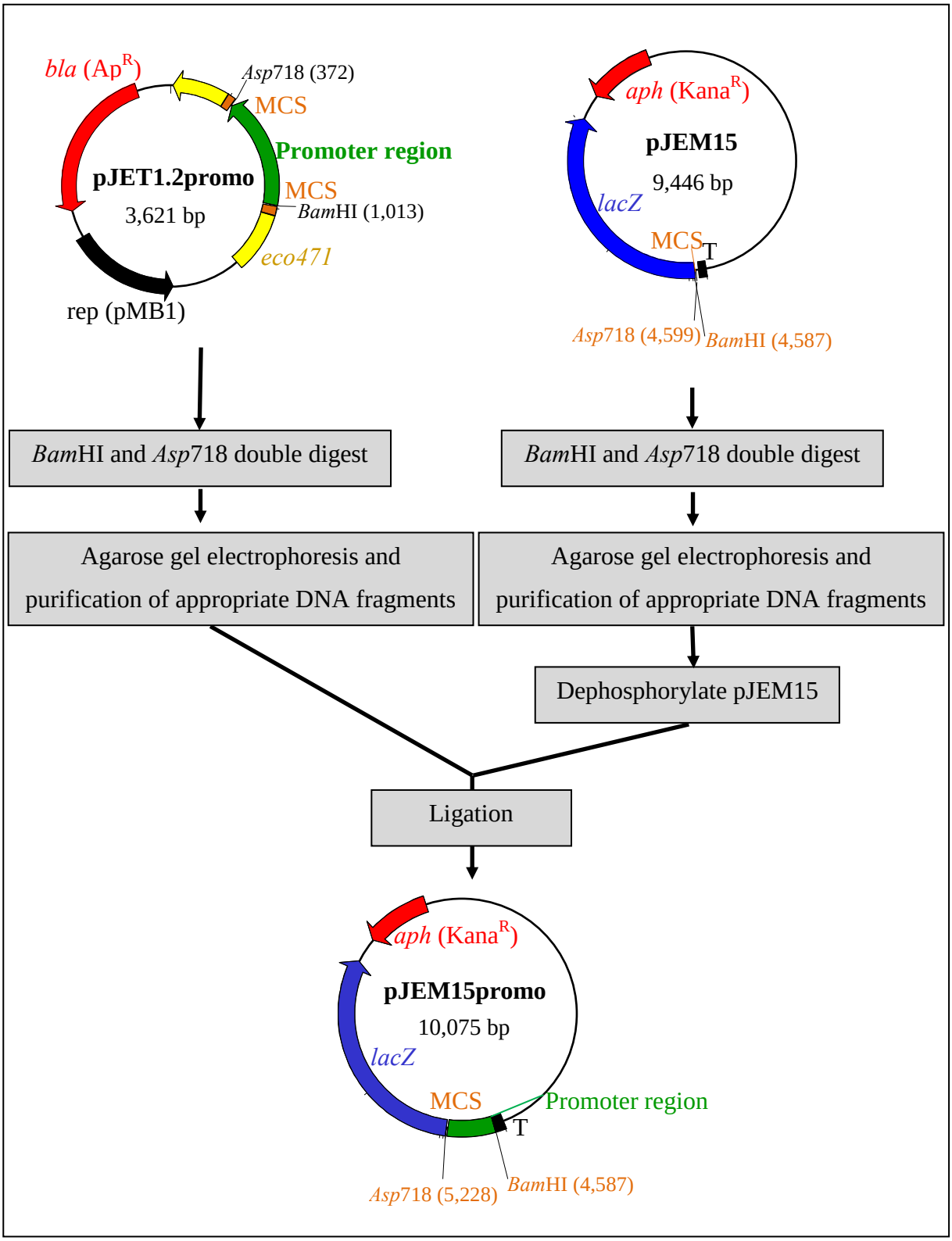


Figure 4.3. Cloning of the promoter region from pJET1.2 into the pJEM15 vector to yield pJEM15promo.

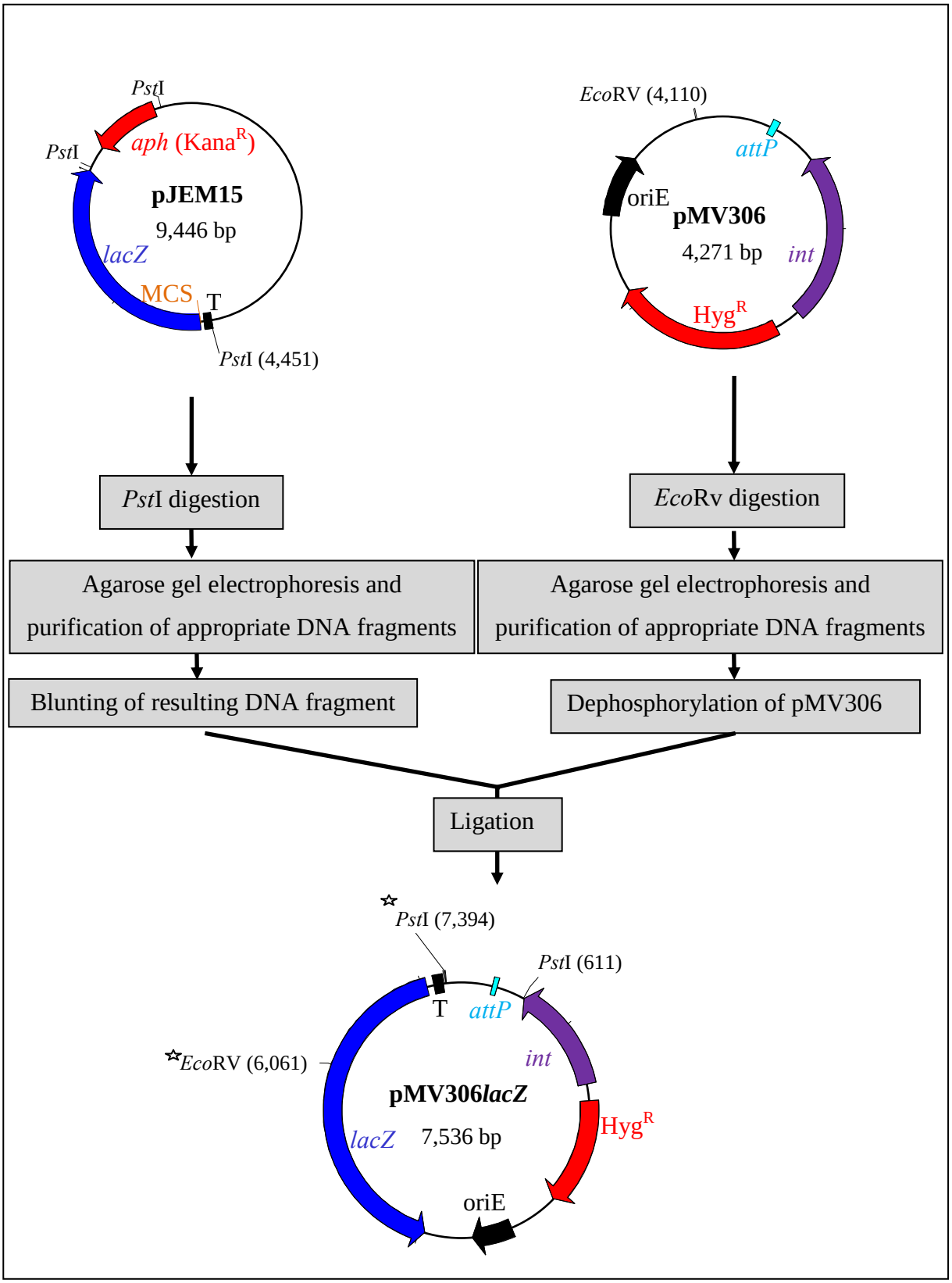


Figure 4.4. Cloning of the promoterless *lacZ* gene from pJEM15 into the integrating vector pMV306 to yield pMV306*lacZ*. ☆ Represents approximate positions since the sequence of vector is not available.

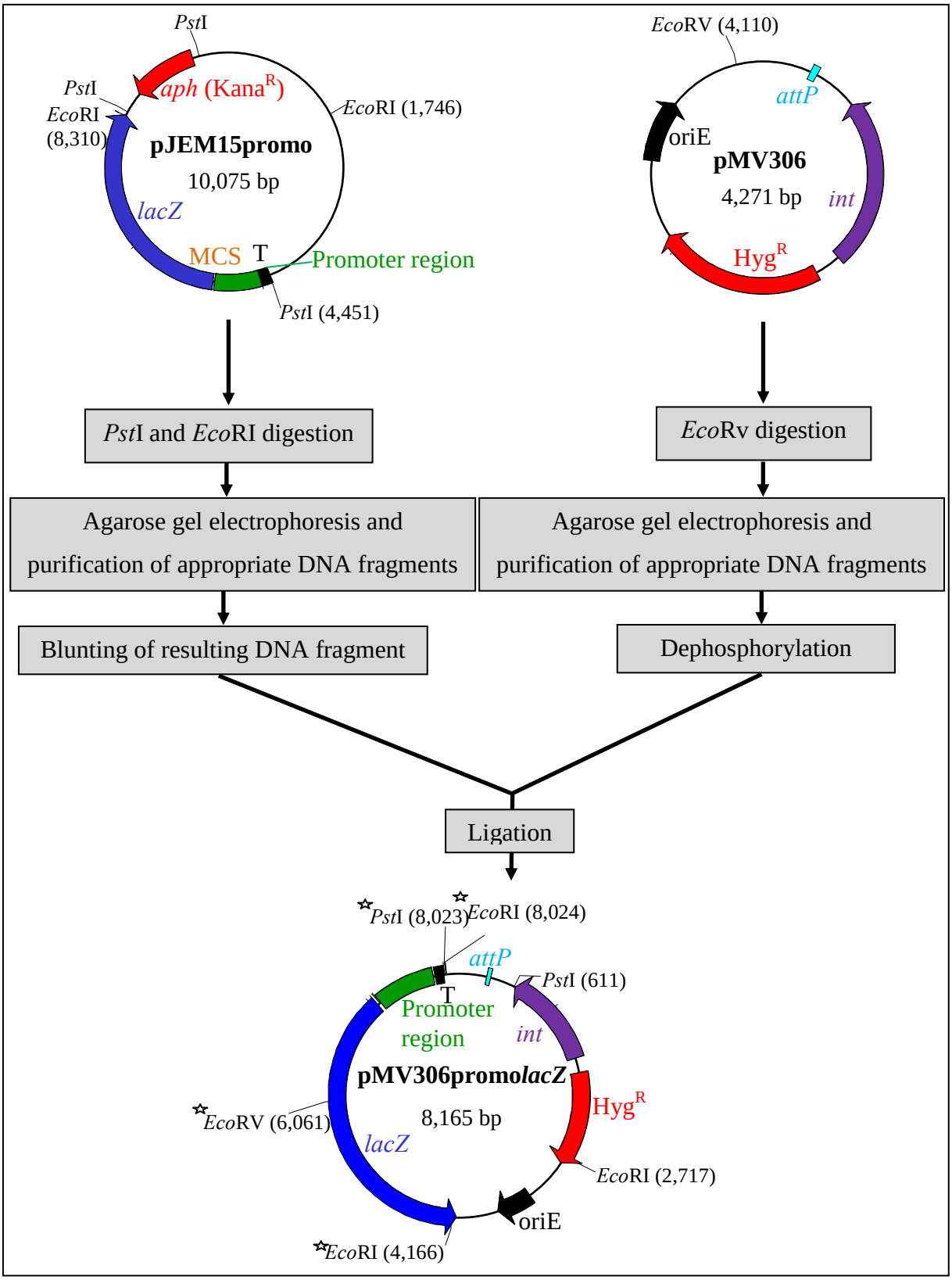


Figure 4.5. Cloning of the promoter region fused to the *lacZ* gene into the integrating vector pMV306 to yield pMV306promo*lacZ*. ☆ Represents approximate positions since the sequence of vector is not available.

4.3 RESULTS

Promoter reporter vectors (episomal and integrating) with the *M. tuberculosis* H37Rv *Rv1258c* promoter upstream of a *lacZ* gene were constructed in order to use for subsequent β-galactosidase assays.

4.3.1 Determining the transcriptional start site of *Rv1258c*

It was necessary to determine the position of the transcriptional start site for *Rv1258c* to verify that it is present in the promoter reporter region that would be cloned into the reporter vectors. In order to determine the position of the transcriptional start site, 5' RACE was done.

M. tuberculosis H37Rv was cultured in Difco Dubos Broth Base (Becton, Dickinson and Company) supplemented with 10% Dubos medium albumin to early log phase. Gene specific primers RACE1 SP1 and RACE1 SP2 (Table 3.4) that bind within *Rv1258c* were used for reverse transcription during PCR amplification during the first 5' RACE experiment. RNA was extracted using the trizol extraction method previously described. The results are summarised in Fig 4.6 as 5' RACE 1.

When examining the PCR product obtained to determine the success of the cDNA synthesis step, the expected PCR product was obtained for the control cDNA (Fig 4.6). However, contamination of the no template control for the PCR and the cDNA PCR reaction was present. Changing all reagents except the primers provided with the kit, did not get rid of the contamination in the no template control. This led us to the conclusion that the control primers used for this reaction were the source of contamination. When the control primers were re-synthesized and the PCR repeated using the newly synthesised control primers, the no template control PCR reaction was clean of any contamination (results not shown).

When testing whether the polyA-tailing step worked effectively, no PCR products were observed after amplification of the control polyA-tailed cDNA (Fig 4.6). The polyA-tailing control step was thus unsuccessful. There was however a product (between 100 and 200 bp in size) observed for the PCR amplifying the polyA-tailed cDNA using the oligo d(T)-anchor primer and gene specific primer (RACE 1SP2) (Fig. 4.6).

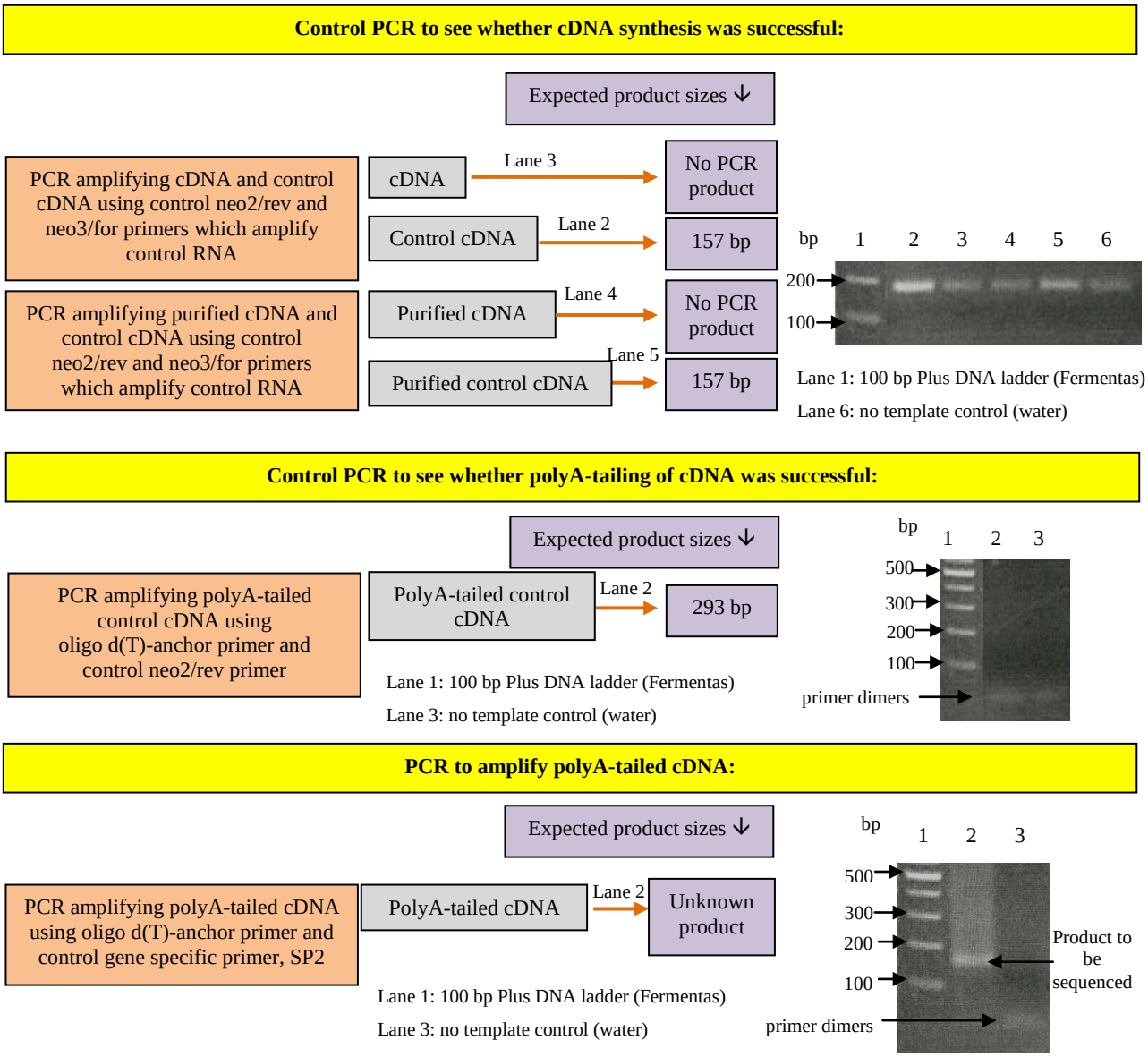


Figure 4.6. Agarose gel electrophoresis of products obtained during PCR during each step of 5' RACE using gene specific primers for *Rv1258c* (5' RACE 1).

This PCR product was cloned into the pGEM-T Easy vector after separation by electrophoresis and the presence of the insert in the vector was confirmed by colony PCR (Fig 4.7). Some of the colonies were picked and the insert sequenced. The PCR product corresponded to 16S rRNA that was amplified presumably because of the high rRNA copy number in comparison to a possible low mRNA copy number of *Rv1258c* transcript. The transcriptional start site could thus unexpectedly not be determined during this 5' RACE (5' RACE 1) experiment.

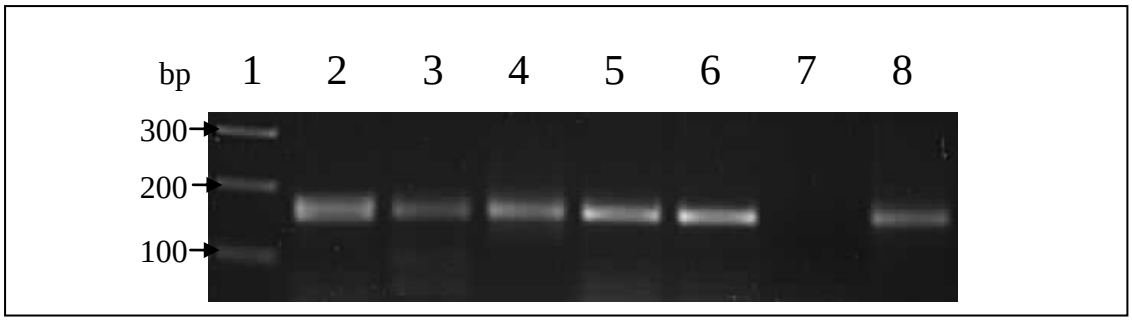


Figure 4.7. Agarose gel electrophoresis of the colony PCR product to confirm the presence of the 5' RACE 1 product in the pGEM-T Easy vector. Lane (1) GeneRuler 100 bp Plus (Fermentas), Lane (2-6) 635 bp product obtained for the colony PCR to check for the presence of the 5' RACE product in five colonies, (7) no template control (water) for the PCR, (8) positive control (purified PCR product) for the PCR.

Since the transcriptional start site could not be determined during this 5' RACE experiment, another strategy using the *Rv1258c* promoter reporter vector constructed during this study would be followed. 5' RACE was repeated using gene specific primers for the *lacZ* gene to determine the position of the transcriptional start site of the *Rv1258c* promoter region cloned into the upstream position of the *lacZ* gene in the multi-copy vector. Since the copy number of the vector is high, enough transcripts of the *Rv1258c* promoter fused to *lacZ* might be produced, which could prevent unwanted non-specific amplification. The second 5' RACE experiment (5' RACE 2) is discussed in Section 4.3.5 after the vector construction sections.

4.3.2 Cloning of *Rv1258c* promoter region into pJET1.2

In order to generate a reporter construct expressing the *Rv1258c* promoter, a 635 bp region directly upstream of the *M. tuberculosis* H37Rv *Rv1258c* gene presumably containing the *Rv1258c* transcriptional start site and promoter, was amplified by PCR using the pRv1258cF and pRv1258cR primers that contained RE cutting sites, *Asp*718 and *Bam*HI. The expected 635 bp product was obtained as shown in Fig 4.8 lane 3 and subsequently cloned into the pJET1.2 sequencing vector to produce pJET1.2promo. RE mapping of pJET1.2promo was used to confirm that the PCR product was successfully cloned into the pJET1.2 vector and that it was cloned into the correct position. Digestion of pJET1.2promo with *Asp*718 and *Bam*HI resulted in two bands of 641 bp and 2,980 bp (Fig 4.9A lane 2), while digestion with *Bgl*II resulted in a 693 bp and a 2,928 bp band (Fig 4.9A lane 3). These were the expected sizes as indicated on the vector map shown in Fig 4.9B confirming that the PCR product was

cloned into the correct position in the pJET1.2 vector. The PCR product present in pJET1.2promo was sequenced and it was confirmed that the 635 bp region upstream of *Rv1258c* was successfully amplified and that no mutations were introduced during amplification.

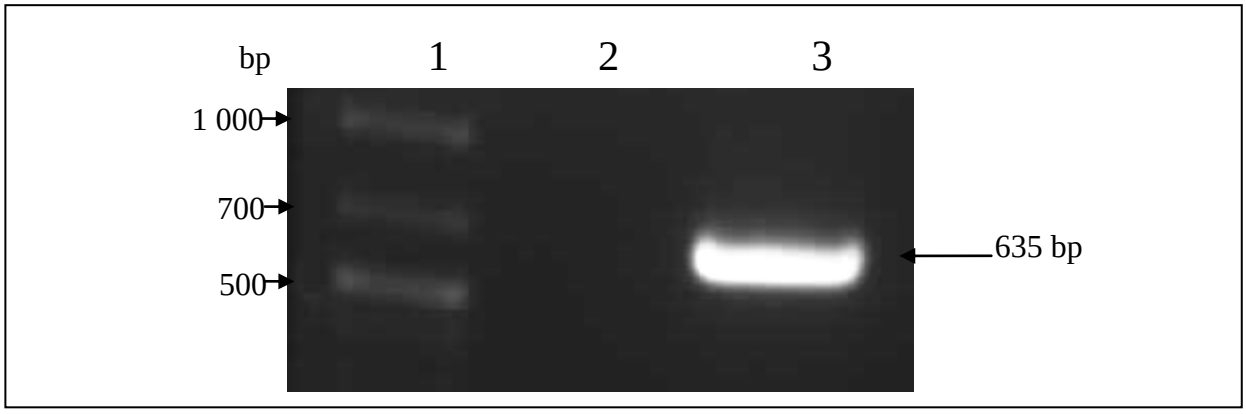


Figure 4.8. Agarose gel electrophoresis of the PCR product when amplifying the 635 bp region upstream of the *M. tuberculosis Rv1258c* gene. Lane (1) GeneRuler 1 kbp Plus DNA Ladder (Fermentas), (2) no template control (water) for the PCR and (3) PCR product.

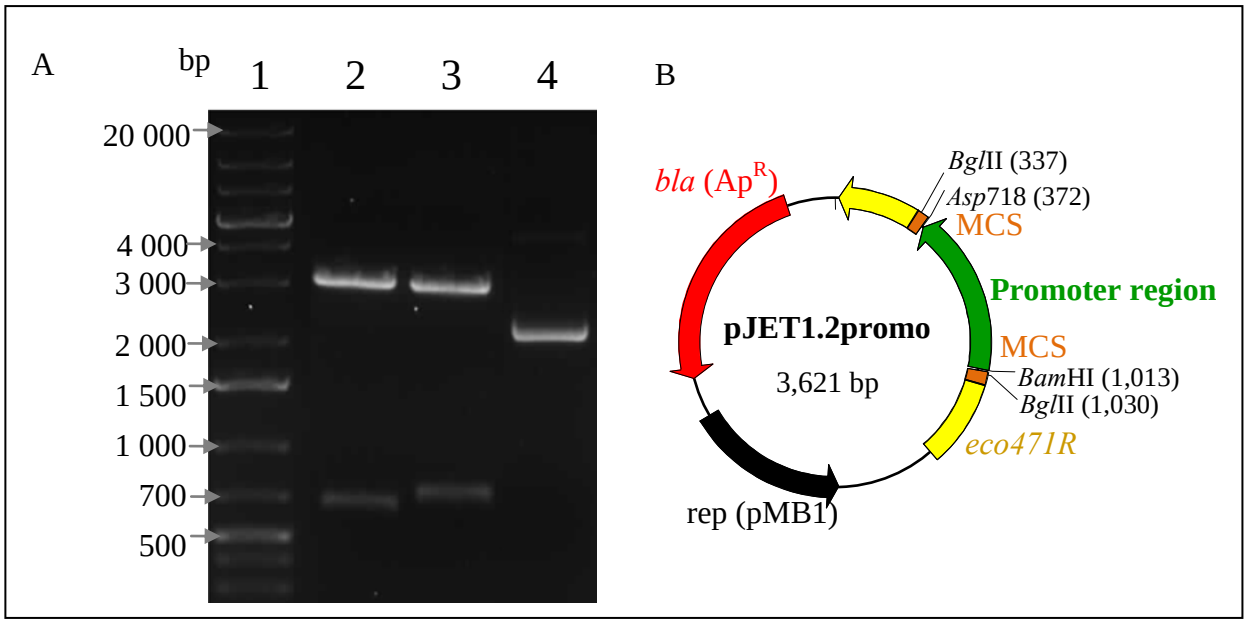


Figure 4.9. (A) Agarose gel electrophoresis of RE mapping of pJET1.2promo. Lane (1) GeneRuler 1 kbp Plus DNA Ladder (Fermentas), pJET1.2promo (505.30 ng) digested with (2) *Asp*718 and *Bam*HI and (3) *Bgl*II and (4) undigested pJET1.2promo, **(B) Vector map of pJET1.2promo indicating RE cutting sites.**

4.3.3 Cloning of *Rv1258c* promoter region into pJEM15

A reporter vector containing the *M. tuberculosis* H37Rv *Rv1258c* promoter region upstream of a reporter gene, a *lacZ* gene, was generated in order to study *Rv1258c* promoter activity using β-galactosidase assays. *Rv1258c* promoter activity serves as an indication of *Rv1258c* gene expression and would be studied using promoter reporter assays. The *Rv1258c* promoter region from pJET1.2promo was cloned into a position upstream of the *lacZ* gene in the pJEM15 vector. The vector is subsequently called pJEM15promo. Colony PCR (using the pRv1258cpF and pRv1258cpR primers) was used to confirm the presence of the pJEM15promo vector in the *E. coli* transformants. A PCR product of 635 bp was observed for all the colonies examined, thereby confirming the presence of promoter region and pJEM15promo in all these colonies (Fig 4.10 lanes 2-10).

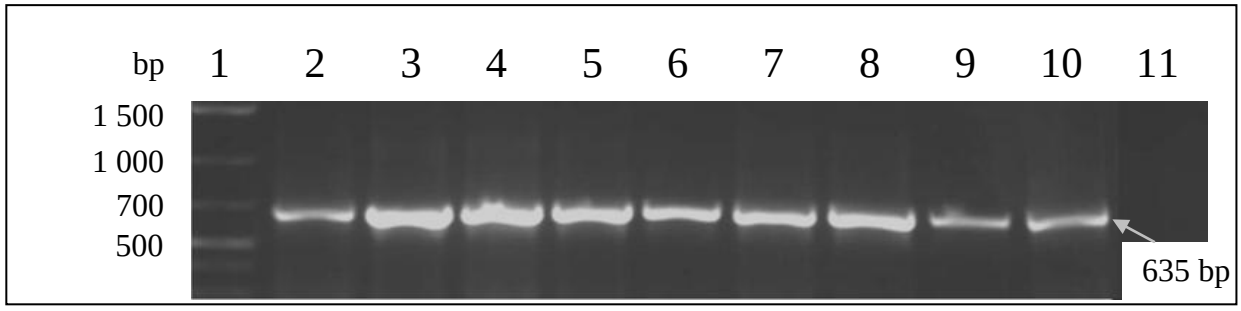


Figure 4.10. Agarose gel electrophoresis of the colony PCR product to confirm the presence of the promoter region in pJEM15promo. Lane (1) GeneRuler 1 kbp Plus (Fermentas), lane (2-10) product obtained for the colony PCR to check for the presence of the promoter region in pJEM15promo for nine colonies (11) no template control (water) for the PCR.

To confirm the presence of the promoter upstream of the *lacZ* gene in pJEM15promo, RE digests of pJEM15 and pJEM15promo were done and the sizes of the resulting DNA fragments examined by agarose gel electrophoresis. The expected sizes of fragments obtained for digestion with *Asp*718 and *Bam*HI were 641 bp and 9,434 bp, for digestion with *Bgl*II were 3,751 bp and 6,324 bp and for digestion with *Bam*HI and *Eco*Rv were 1,828 bp and 8,247 bp (Fig 4.11B). The DNA fragments obtained during restriction mapping were all the expected sizes (Fig 4.11A). Notice the shift of approximately 641 bp of the second largest band in the *Pst*I digested pJEM15promo vector (Fig 4.11A lane 4) in comparison to the pJEM15 vector (Fig 4.11A lane 3). There is also an upward shift of approximately 641 bp in the largest band in the *Pst*I and *Eco*RI digested pJEM15promo (Fig 4.11A lane 6) in

comparison to the pJEM15 vector (Fig 4.11A lane 5). Three bands of approximately the sizes expected for *Hind*III digested pJEM15promo were observed during RE mapping (Fig 4.11A lane10). The digests thus indicate that the promoter region was successfully cloned into the correct position (upstream of the *lacZ* gene) into the pJEM15 vector to obtain pJEM15promo.

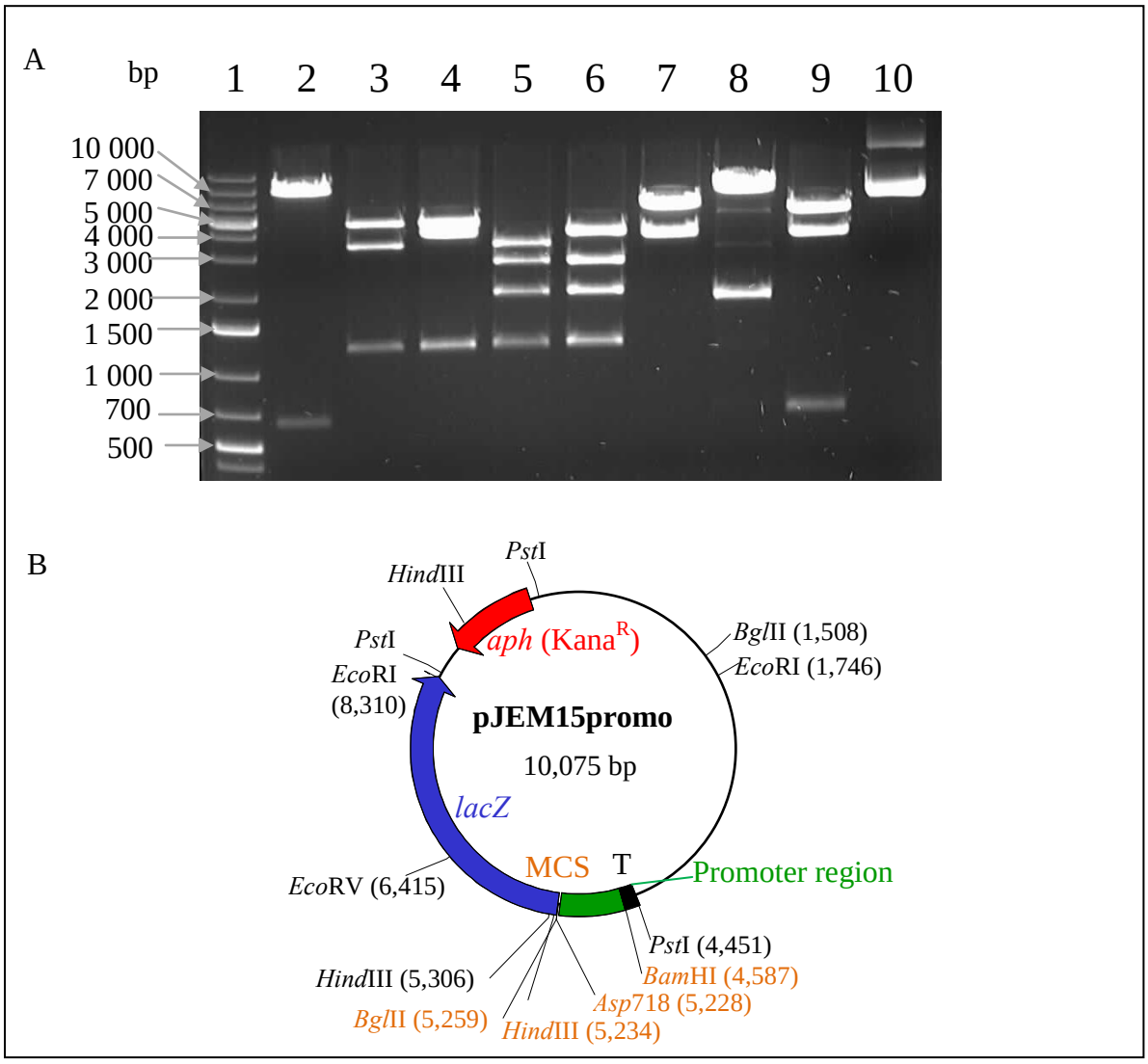


Figure 4.11. (A) Agarose gel electrophoresis of RE mapping of pJEM15 and pJEM15promo. Lane (1) GeneRuler 1 kbp Plus DNA Ladder (Fermentas), pJEM15promo (595.10 ng) digested with (2) *Asp*718 and *Bam*HI, (4) *Pst*I, (6) *Pst*I and *Eco*RI, (7) *Bgl*II, (8) *Bam*HI and *Eco*Rv and (9) *Bam*HI and *Hind*III, pJEM15 (525.48 ng) digested with (3) *Pst*I and (5) *Pst*I and *Eco*RI and (10) undigested pJEM15promo, **(B) Vector map of pJEM15promo.**

4.3.4 Construction of integrating vectors

To ensure that the copy number of the episomal vector system does not affect the experiment and to improve the vector stability in the absence of the selectable antibiotic, integrating vectors were constructed. The pMV306 integrating vector contains an integrase gene that enables it to integrate into the *attB* site of the mycobacterial genome. The promoterless *lacZ* gene from pJEM15 and the promoter fused to the *lacZ* gene from pJEM15promo respectively, were ligated into pMV306 to create the integrating vectors subsequently called pMV306*lacZ* and pMV306promo*lacZ* respectively. Because of blunting of the RE digested fragment containing the *lacZ* gene and the *Rv1258c* promoter region fused to the *lacZ* gene, the fragment could be inserted in either orientation into the pMV306 vector.

RE mapping was used to determine whether the fragments were correctly cloned into pMV306 and to determine the orientation in which the fragments were inserted. The expected sizes of fragments obtained for digestion of pMV306*lacZ* with *Bgl*II were approximately 3,124 bp, 3,023 bp and 1,389 bp for the fragment cloned into the pMV306 in a reverse orientation (Fig 4.12B). These fragment sizes were obtained (Fig 4.12A lane 2). The expected sizes of fragments obtained for digestion with *Pst*I were approximately 6,783 bp and 753 bp (Fig 4.12B) and were obtained for the RE mapping (Fig 4.12A lane 3). The expected sizes of fragments obtained for digestion with *Bam*HI were approximately 6,703 bp, 631 bp and 122 bp and those for *Asp*718 were approximately 4,375 bp and 3,161 bp (Fig 4.12B lane 4 and 7) The DNA fragments obtained during RE mapping were all the expected sizes and were that which was expected for the *lacZ* gene being cloned in a reverse direction into the pMV306 vector to obtain pMV306*lacZ* (Fig 4.12). This confirmed that the promoterless *lacZ* gene was correctly cloned into pMV306 in a reverse orientation.

The expected sizes of fragments obtained for digestion of the pMV306promo*lacZ* vector with *Asp*718 were approximately 5,004 bp and 3,161 bp (Fig 4.13A lane 2) and those expected for *Bgl*II were approximately 3,124 bp, 3,023 bp and 2,018 bp (Fig 4.13A lane 3). The expected sizes of fragments obtained for digestion with *Eco*Rv were approximately 8,165 bp (Fig 4.13A lane 4) and those for *Pst*I were approximately 7,412 bp and 753 bp (Fig 4.13A lane 5) and for *Hind*III were approximately 7,304 bp, 789 bp and 72 bp (Fig 4.13A lane 6). These sizes were those expected for the cloning of the *Rv1258c* promoter region fused to *lacZ*

into pMV306 in a reverse orientation (Fig 4.13B). This confirmed that the promoter fused to the *lacZ* gene was correctly cloned into pMV306 in a reverse orientation.

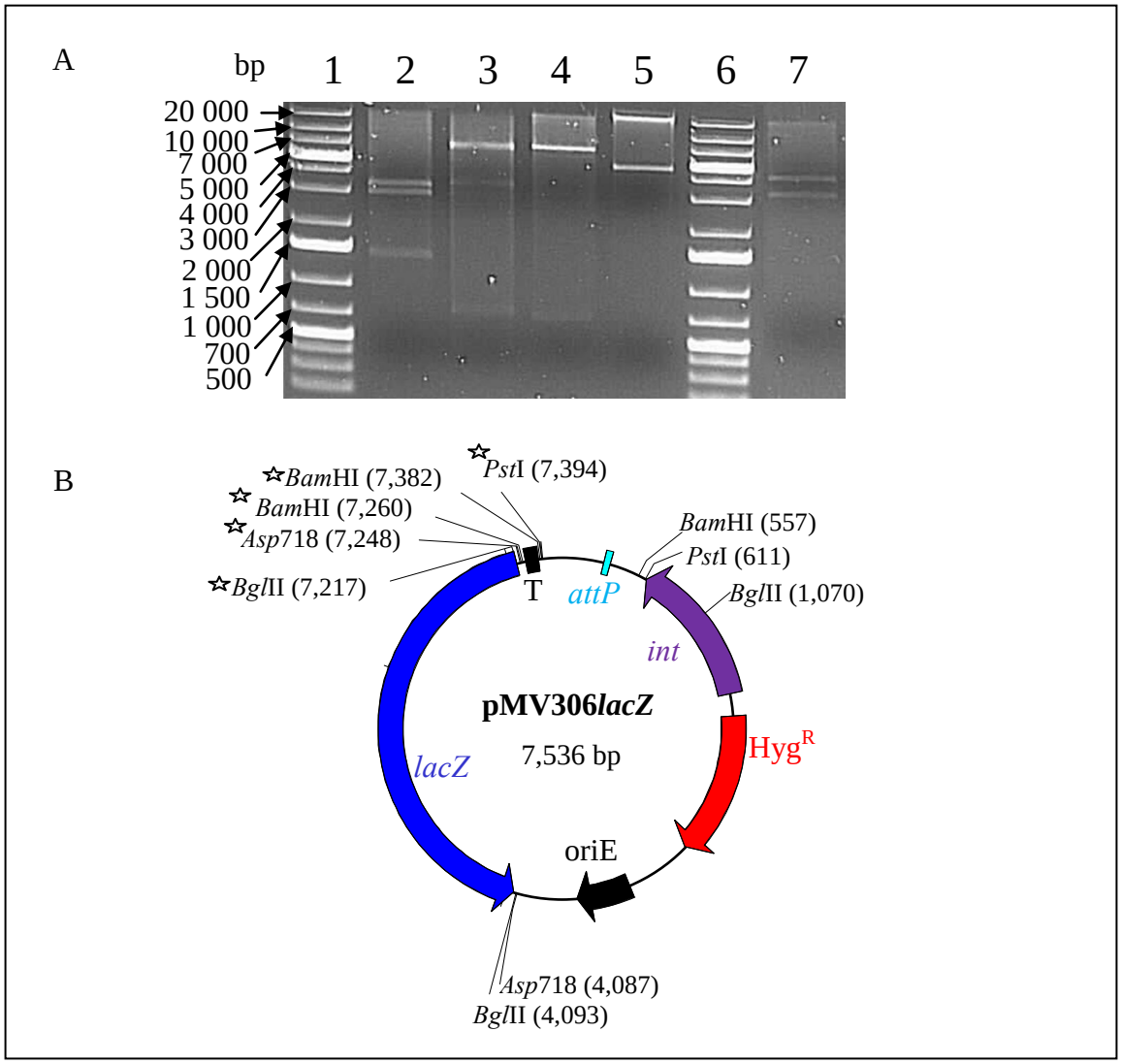


Figure 4.12. (A) Agarose gel electrophoresis of RE mapping of pMV306*lacZ*. Lane (1 and 6) GeneRuler 1 kbp Plus DNA Ladder (Fermentas), pMV306*lacZ* (463.58 ng) digested with (2) *Bgl*II, (3) *Pst*I, (4) *Bam*HI, and (7) *Asp*718 and (5) undigested pMV306*lacZ*, **(B) Vector map of pMV306*lacZ*.** ☆ Represents approximate positions since the sequence of vector is not available.

4.3.5 5' RACE repeated using constructed vector

Since the 5' RACE experiment was previously unsuccessful (see 5' RACE 1) it was repeated using the pJEM15promo transformed strain and three gene specific primers, which bind to the *lacZ* gene in an attempt to ensure that a higher copy number of the *Rv1258c* promoter

transcript would overcome non-specific amplification. The experimental results are referred to as 5' RACE 2.

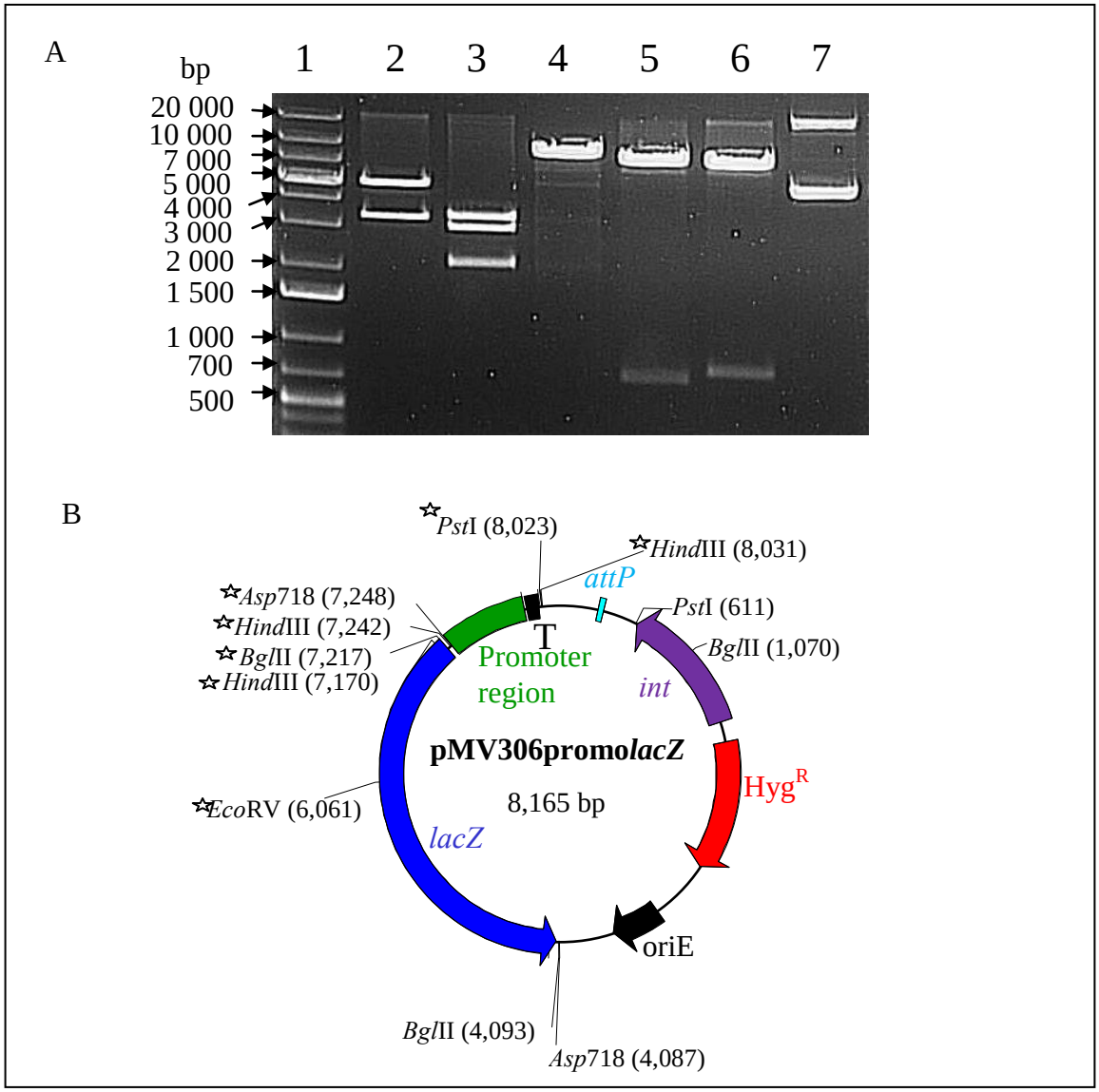


Figure 4.13. (A) Agarose gel electrophoresis of RE mapping of pMV306promo*lacZ*. Lane (1) GeneRuler 1 kbp Plus DNA Ladder (Fermentas), pMV306promo*lacZ* (555.61 ng) digested with (2) *Asp*718 and (3) *Bgl*II and (4) *Eco*Rv (5) *Pst*I (6) *Hind*III and (7) undigested pMV306promo*lacZ*, **(B) Vector map of pMV306promo*lacZ*.** ☆ Represents approximate positions since the sequence of vector is not available.

M. tuberculosis H37Rv (pJEM15promo) was cultured to an OD of approximately 0.80 at 600 nm. RNA was extracted using the RNA ProBlue kit as previously described with a few minor alterations: After the treatment with DNase I, the RNA was precipitated and resuspended in water. The sample was treated with Turbo DNase as previously described.

DNase I inactivation reagent from the TURBO DNA-free kit (Applied Biosystems, Ambion) was used according to the manufacturer's instructions, to inactivate the Turbo DNase. The RNA was then precipitated overnight and re-suspended in water.

Three gene specific primers RACE2 SP1, RACE2 SP2 and RACE2 SP3 which bind within the *lacZ* gene of the pJEM15promo vector (which has the promoter region of *Rv1258c* cloned into an upstream position) was used to determine the position of the *Rv1258c* transcriptional start site using 5' RACE. The PCR product obtained after the amplification of polyA-tailed cDNA with the oligo d(T)-anchor primer, RACE2 SP2, was re-amplified using a second nested primer, RACE2 SP3.

The results for 5' RACE 2 are summarized in Fig 4.14. When examining the PCR product to determine the success of the cDNA synthesis, the expected PCR product was obtained for the control cDNA. The use of re-synthesised control primers as opposed to those included in the kit was again required to obtain clean no template controls because of contamination of the control primers of the new kit used (Fig 4.14). Amplification of the control cDNA produced the expected 157 bp product and no product was obtained upon amplification of the cDNA or for the no template control (Fig 4.14). A PCR product of the expected size, 293 bp, was observed after amplification of the control polyA-tailed cDNA, confirming that the polyA-tailing step was successful. There was however no product observed for the PCR to amplify polyA-tailed cDNA (Fig 4.14) and therefore a re-amplification of the latter PCR product using the PCR anchor primer and a second nested gene specific primer RACE2 SP3 was done.

A PCR product of between 100 and 200 bp in size was obtained upon re-amplification. Since a smear was observed on the gel surrounding this band (Fig 4.14), we decided to purify the PCR product without prior separation using gel electrophoresis in order to keep possible rare transcripts since the size of the product obtained for 5' RACE2 was similar to that of 5' RACE1 where 16S rRNA was previously amplified. The purified PCR product was cloned into the pGEM-T Easy vector, *E. coli* was transformed with these vectors and the presence of the insert in the vector was confirmed by colony PCR using the PCR anchor and the RACE2 SP3 primers (Fig 4.15).

Some of the colonies contained inserts of different sizes (Fig 4.15). Colonies with a range of insert sizes were picked and the inserts sequenced. The transcriptional start site was

determined to be only six base pairs upstream of the *Rv1258c* gene start codon (Fig 4.16). The sequencing product of the RACE product aligned to a region of the pJEM15promo vector sequence is indicated in Addendum A.

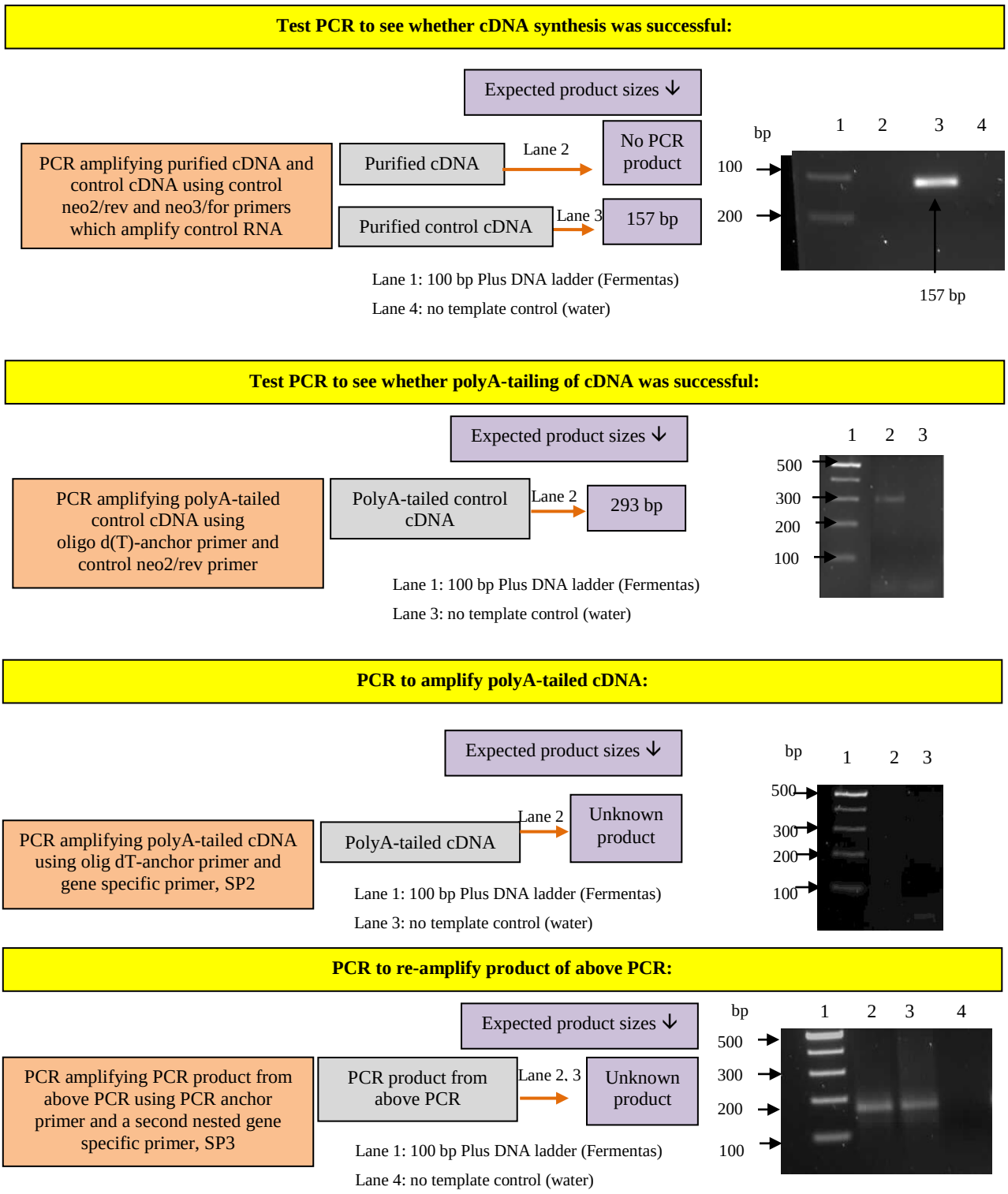


Figure 4.14. Agarose gel electrophoresis of products obtained during PCR during each step of 5' RACE (5' RACE 2).

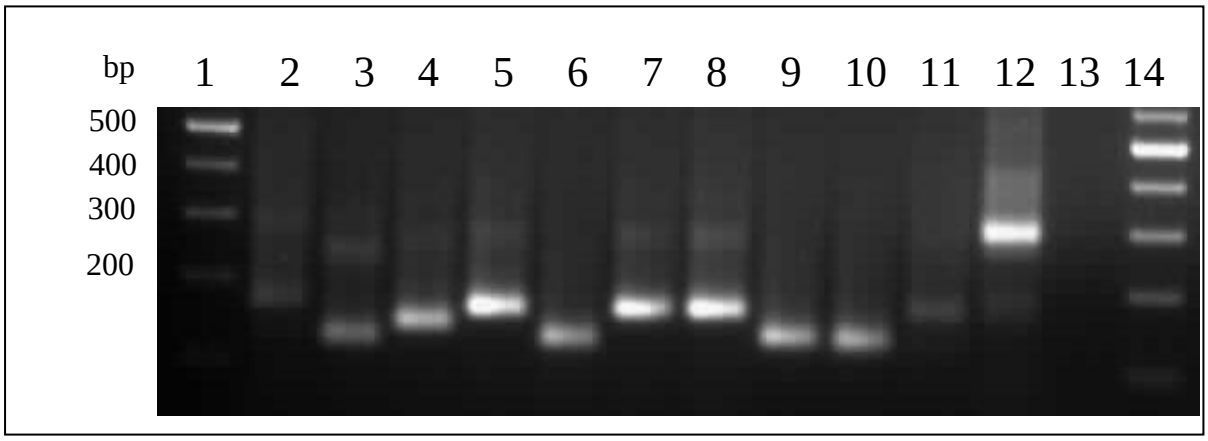


Figure 4.15 Agarose gel electrophoresis of the colony PCR product to confirm the presence of the 5' RACE 2 product in the pGEM-T Easy vector using the PCR anchor primer and the gene specific primer, RACE2 SP3. Lane (1) and (14) 100 bp Plus GeneRuler (Fermentas), lanes (2-12) colonies 2-12 and lane (13) no template control for the PCR.

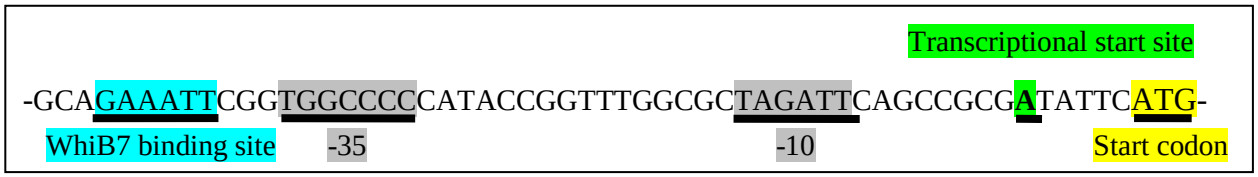


Figure 4.16. Position of the transcriptional start site relative to the start codon of *Rv1258c* in *M. tuberculosis*. The position of the -10 Pribnow box and -35 promoter regions and the possible predicted WhiB7 binding site according to Burian *et al.*, (2012) are indicated.

4.4 DISCUSSION

In order to construct promoter reporter vectors with the promoter region of *M. tuberculosis* H37Rv *Rv1258c*, it was necessary to determine the position of the transcriptional start site of *Rv1258c*. This would give us an idea of where the promoter is located. After performing 5' RACE twice, the transcriptional start site was determined to be at a position only six nucleotides upstream of the start codon of *Rv1258c*. In agreement with this finding, the transcriptional start site is generally located five to nine base pairs downstream of the -10 promoter region and the first nucleotide can be any one of the four bases (Newton-Foot & Gey van Pittius, 2012). Promoter regions generally have a -10 and -35 promoter region. The -10 promoter region is essential for transcription, as the sigma factors need to bind in that position. The -35 promoter region is sometimes non-essential for transcription in

M. tuberculosis, but promoters lacking this could need addition transcriptional activators in order for transcription to be initiated. Promoters are generally a minimum of 40 bp in size, but could be as big as 2 kbp (Newton-Foot & Gey van Pittius, 2012). Generally, promoters contain binding sites for regulators involved in regulating the expression of the downstream gene (Newton-Foot & Gey van Pittius, 2012). The *whiB7* gene encodes a global transcriptional regulator which also regulates *Rv1258c* (Morris et al., 2005). Analysis of the promoters of genes regulated by WhiB7 led to the identification of *whiB7* promoter motifs. This promoter motif was identified upstream of *Rv1258c* at position -39 to -45 upstream of the start codon of *Rv1258c* (Burian *et al.*, 2012).

The position of the transcriptional start site at six nucleotides upstream of the start codon was however questioned since there was no apparent ribosome binding site between the start codon and the determined transcriptional start site. Ribosome binding sites are generally adenosine and guanine rich region of at least four base pairs (Newton-Foot & Gey van Pittius, 2012). In some cases, the transcriptional start site and the start codon are present close to each other and there are no ribosome binding sites between them. The mRNA is leaderless in such cases (Newton-Foot & Gey van Pittius, 2012). It could be that the *Rv1258c* mRNA is leaderless and that is why no apparent ribosome binding site is present. Interestingly, another group had previously also determined that the transcriptional start site for *Rv1258c* was only a very few base pairs upstream of the gene also using 5' RACE, but dismissed these results (Villellas, 2012). Although we determined the position of the transcriptional start site, the results were not conclusive.

An episomal vector with the promoter region of *M. tuberculosis* H37Rv *Rv1258c* upstream of a *lacZ* gene (pJEM15promo) was successfully constructed to be used for studying *Rv1258c* promoter activity using β-galactosidase assays. This episomal vector would need to be selected by adding kanamycin to the media and kanamycin could possibly influence *Rv1258c* promoter activity. The episomal vector is also a multi-copy vector and this could influence the functioning or binding of transcription factors to the promoter region and thus have an impact on promoter activity. We therefore chose to modify the pJEM15 system to integrate into the mycobacterial genome at the *attB* site. RE digests and PCR was used to confirm that the vectors were correctly constructed. However, the latter does not prove the utility of the vectors in mycobacteria. We therefore needed to determine the functioning of the episomal and integrating systems in the closely related, fast-growing, non-pathogenic species

M. smegmatis mc^2155, before choosing which vector system to use for subsequent studies in *M. tuberculosis*. Promoter reporter vectors (episomal and integrating), were successfully constructed in order to use for investigating *M .tuberculosis Rv1258c* promoter expression using β-galactosidase assays.

CHAPTER 5: INVESTIGATING THE FUNCTIONAL CONSEQUENCES OF THE EPISOMAL AND INTEGRATING VECTOR SYSTEMS AND THE EFFECTS OF *RPOB* MUTATIONS ON THE *M. TUBERCULOSIS RV1258C* PROMOTER ACTIVITY IN *M. SMEGMATIS*

5.1 INTRODUCTION

The episomal and integrating vector functioning was investigated in *M. smegmatis* mc^2155. *M. smegmatis* is a closely related species to *M. tuberculosis* and has long been used as model organism for studying relevant basic cellular processes and gene expression relevant to *M. tuberculosis*. *M. smegmatis* is a fast-growing non-pathogenic mycobacterial species that is present in soil. This makes it ideal to investigate the vector functioning since results would be obtained fast (within three to four days) as opposed to one to two weeks if *M. tuberculosis* was used. In addition, working with *M. smegmatis* does not require working under Biohazard safety level three conditions. Vector functioning was thus assessed in *M. smegmatis*.

Previous studies showed a 79% protein homology between *M. smegmatis* and *M. tuberculosis* (Wang *et al.*, 2005). This implies that the expressed proteome of *M. smegmatis* is highly relevant to that of *M. tuberculosis* (Wang *et al.*, 2005). *M. smegmatis* has many genes encoding putative drug efflux pumps that are homologues to those genes encoding pumps found in *M. tuberculosis* (Li *et al.*, 2004). Bioinformatic searches revealed that *Rv1258c* homologue, *MSMEG_5033*, in *M. smegmatis* mc^2155 is a pseudogene (SmegmaList). When aligning parts of *Rv1258c* with *MSMEG_5033*, a 67% amino acid residue identity with that of *Rv1258c* was identified based on bioinformatic searches (Li *et al.*, 2004).

Comparison of the *rpoB* nucleotide sequence of *M. tuberculosis* and *M. smegmatis*, revealed a high degree of similarity (Hetherington *et al.*, 1995; Kim *et al.*, 1999), which means that *M. smegmatis* can serve as a model for studying the effects of *rpoB* mutations on *M. tuberculosis Rv1258c* promoter expression using β-galactosidase assays. In addition to determining the functional consequences of the vector systems, the effects of *rpoB* mutations on *Rv1258c* promoter activity in *M. smegmatis* would thus also be studied.

5.2 METHODOLOGY

5.2.1 Growth curve analysis for *M. smegmatis* mc^{2}155

Growth curves were generated for all the *M. smegmatis* mc^{2}155 wild-type and *rpoB* mutants transformed with the episomal and integrating vector systems. The pre-cultures were sub-cultured into 100 ml 7H9 GS containing the appropriate antibiotics to an OD of 0.02 at 600 nm. The OD at 600 nm was measured every three hours (representing the doubling time of *M. smegmatis* mc^{2}155) for 30 hours using an Ultrospec 4051 (LKB Biochrom).

5.2.2 *Rv1258c* promoter activity in the absence of rifampicin

The *M. smegmatis* wild-type and *rpoB* mutants harbouring the episomal and integrating vectors respectively, were cultured (100 ml) overnight to early log phase. Cultures contained the selectable antibiotic kanamycin and hygromycin respectively. WCLs were prepared at early log phase and three hours later for non-exposure experiments. The protein concentration was determined and the promoter activity was determined using β-galactosidase assays. The experiment was done in triplicate and the β-galactosidase activity for the three experiments averaged.

5.2.3 *Rv1258c* promoter activity in response to rifampicin

M. smegmatis mc^{2}155 *rpoB* mutants harbouring the episomal and integrating vectors respectively, were cultured (100 ml) overnight to early log phase. Cultures contained the selectable antibiotic kanamycin and hygromycin respectively. Each culture was divided in two, one of which was exposed to 10 µg/ml RIF and the other to DMSO (diluent for RIF). Exposure to DMSO was done to determine the RIF-specific response. WCLs were prepared as previously described after one, three and six hours exposure to RIF. The protein concentration was determined and *Rv1258c* promoter activity was determined using β-galactosidase assays. The experiment was done in triplicate and the β-galactosidase activity for the three experiments averaged.

5.3 RESULTS

The functioning of the episomal and integrating vectors were tested using β-galactosidase assays in *M. smegmatis* in order to determine which vector system would be used for subsequent β-galactosidase assays in *M. tuberculosis*. The effects of *rpoB* mutations on the *M. tuberculosis Rv1258c* promoter expression was also investigated in *M. smegmatis*. This was done in order to compare the results to those of β-galactosidase assays in *M. tuberculosis*, thereby assessing the use of *M. smegmatis* as model for investigating the expression of the *M. tuberculosis Rv1258c* promoter.

5.3.1 Growth curve analysis for *M. smegmatis*

Growth curves were generated for all the *M. smegmatis* mc^2155 wild-type and *rpoB* mutants harbouring the episomal and integrating vector systems. This was done in order to determine whether the presence of the vectors influenced the growth of the bacteria and to determine the effects of the *rpoB* mutations on the growth of the *M. smegmatis rpoB* mutants. The OD at which the log growth phase was reached was also determined using the growth curves since the WCLs for the β-galactosidase assays would be prepared at early log phase.

When comparing the growth of the same strain harbouring the pJEM15 and pJEM15promo vectors respectively, (*M. smegmatis* mc^2155 wild-type (pJEM15) with *M. smegmatis* mc^2155 wild-type (pJEM15promo)), no differences in the growth were observed (Fig 5.1). Comparison of the growth of *M. smegmatis* mc^2155 wild-type and S531L *rpoB* mutant harbouring the pJEM15promo vectors, showed ($p<0.05$) differences in growth at 18 ($p=0.0395$), 21 ($p=0.0048$) and 24 ($p=0.0387$) hours of growth (representing the log growth phase) (Fig 5.1). Similarly the growth of *M. smegmatis* mc^2155 wild-type and H526Y *rpoB* mutant harbouring the pJEM15promo vectors, was significantly ($p<0.05$) different at 18 ($p=0.0235$), 21 ($p=0.0017$) and 24 ($p=0.0069$) hours of growth (representing the log growth phase) (Fig 5.1). A significant difference in growth was also present at 24 hours ($p=0.0150$) between the *M. smegmatis* mc^2155 S531L and H526Y *rpoB* mutants (Fig 5.1). In general, the mutants tended to grow slower than the wild-type strains. All the strains were in early log growth phase at an OD of approximately 0.50 to 0.70 at 600 nm (Fig 5.1).

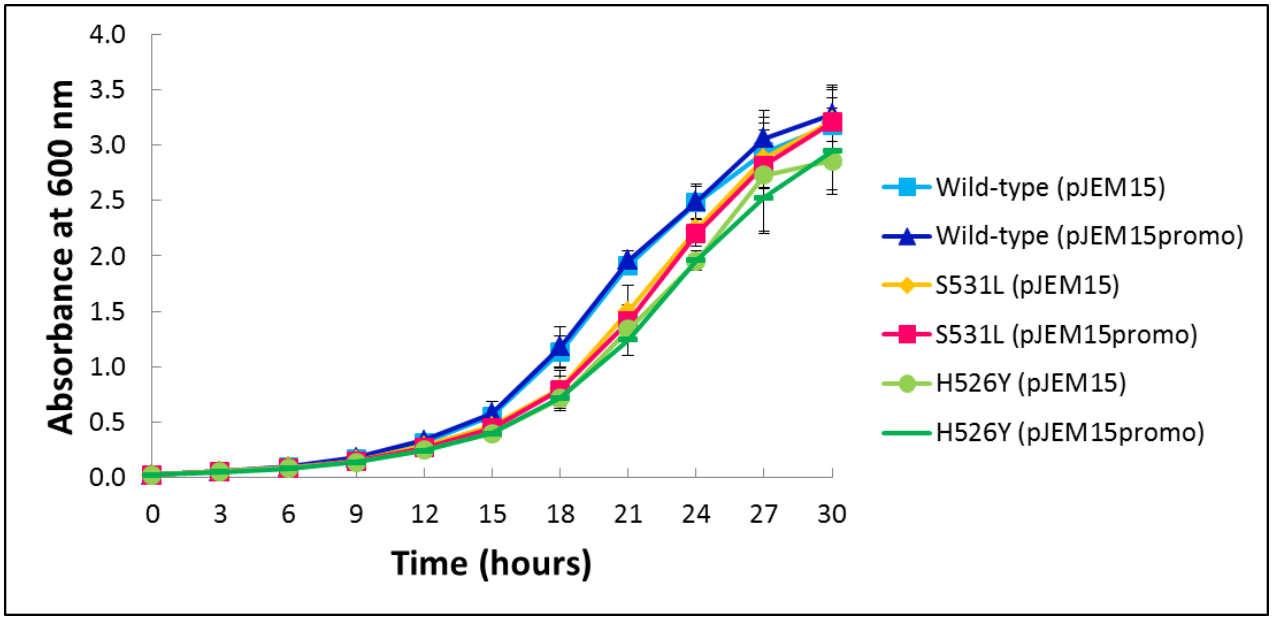


Figure 5.1. Growth curve for *M. smegmatis* mc^{2}155 wild-type and S531L and H526Y *rpoB* mutants transformed with the episomal vectors, cultured in 7H9 GS containing 50 µg/ml kanamycin. The graph represents the average of three independent experiments and error bars represent standard deviation. Statistically significant difference in growth are not indicated on the graph but are discussed in the text.

Similarly, when comparing the growth of the same strain transformed with either the pMV306*lacZ* or pMV306promo*lacZ* vectors, (for example *M. smegmatis* mc^{2}155 wild-type *attB*::pMV306*lacZ* with *M. smegmatis* mc^{2}155 wild-type *attB*::pMV306promo*lacZ*) no differences in the growth were observed (Fig 5.2). Significant differences in growth were observed between the *M. smegmatis* mc^{2}155 wild-type and *rpoB* S531L mutant strains harbouring the pMV306promo*lacZ* vector at 15 (p=0.0276) and 18 (p=0.0183) hours of growth. There was also a significant difference in the growth of *M. smegmatis* mc^{2}155 wild-type and H526Y *rpoB* mutant harbouring the pMV306promo*lacZ* vector at 18 hours (p=0.0413) of growth (Fig 5.2). There were no significant differences in growth between the two *rpoB* mutants. All the strains were in early log growth phase at an OD of approximately 0.50 to 0.70 at 600 nm.

5.3.2 *Rv1258c* promoter activity in the absence of rifampicin

Rv1258c promoter activity was investigated using the episomal vector system and the integrating vector system in *M. smegmatis* mc^{2}155 to validate the vectors that were developed

and to determine which system should be used for subsequent β-galactosidase assays in *M. tuberculosis*. The effect of *rpoB* mutations on *Rv1258c* promoter activity in *M. smegmatis* was also investigated. *M. smegmatis* was cultured overnight until early log phase (an OD between 0.50 and 0.70 as determined during growth curve analyses) and WCLs were prepared at early log phase and three hours later. An example of a kinetic curve generated for β-galactosidase assays is given in Addendum B. The β-galactosidase activity was determined for a range of different WCL protein concentrations. An appropriate protein concentration and reaction incubation period was selected (refer to Addendum B for an explanation of how this was done). The final protein concentration of WCLs used for the calculation of the β-galactosidase activity was 9.6 µg/ml for the episomal vector system and 153.6 µg/ml for the integrating vector system. The absorbance reading for the enzymatic reaction at 35 min exposure of the enzyme (β-galactosidase) to the substrate ONPG was chosen to calculate the β-galactosidase activity.

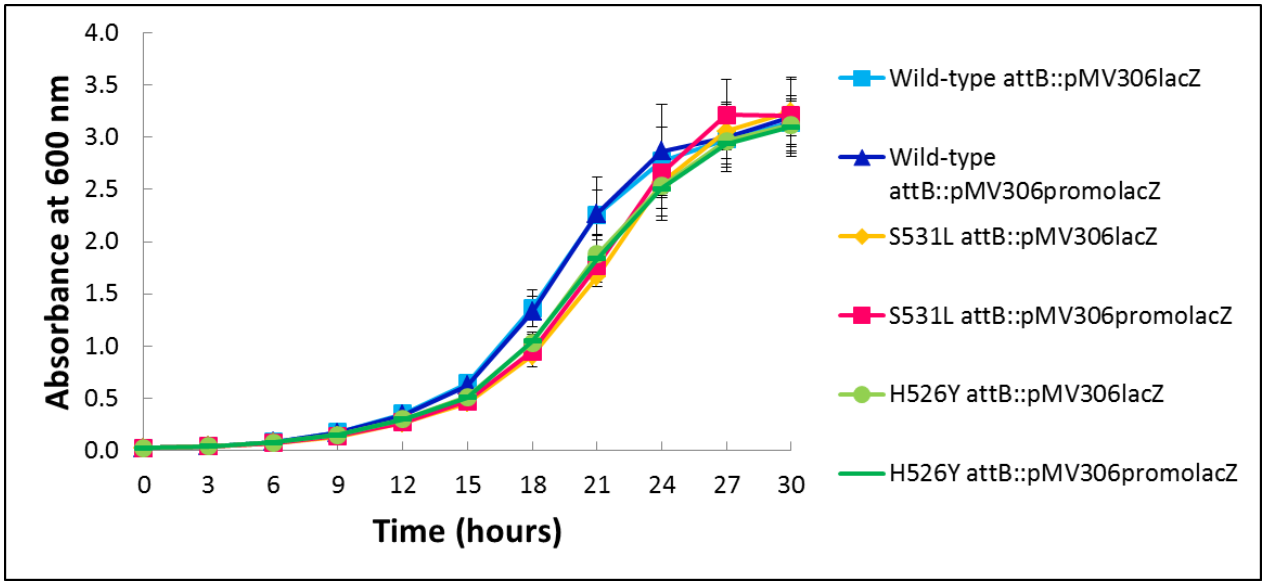


Figure 5.2. Growth curve for *M. smegmatis* mc^{2}155 wild-type and S531L and H526Y mutants harbouring the integrating vectors in 7H9 GS containing 50 µg/ml hygromycin. The graph represents the average of three independent experiments and error bars represent standard deviation. Statistically significant difference in growth are not indicated on the graph but are discussed in the text.

The β-galactosidase activity of the strains transformed with pJEM15 and pMV306*lacZ* vectors indicates the background activity in the absence of the promoter. This background activity was negligible for both vector systems as can be seen in Fig 5.3 and Fig 5.4. This was

expected as there is a transcriptional terminator and no promoter present upstream of the *lacZ* gene in the pJEM15 and pMV306*lacZ* vectors. This proves that the transcriptional terminator is functional in both vector systems and that background activity will not affect the experimental results in either of the vector systems.

The *M. tuberculosis* H37Rv *Rv1258c* promoter activity in *M. smegmatis* mc^2155 *rpoB* S531L (pJEM15promo) was approximately half that of the *M. smegmatis* mc^2155 wild-type (pJEM15promo) (Fig 5.3), while the *Rv1258c* promoter activity in *M. smegmatis* mc^2155 *rpoB* S531L *attB*::pMV306promo*lacZ* was significantly lower at approximately 1.45 times less than that of the *M. smegmatis* mc^2155 wild-type *attB*::pMV306promo*lacZ* (Fig 5.4). The results obtained with the episomal and integrating vector systems were thus comparable.

The *M. tuberculosis* H37Rv *Rv1258c* promoter activity in *M. smegmatis* mc^2155 *rpoB* H526Y (pJEM15promo) was not significantly different to that of the *M. smegmatis* mc^2155 wild-type (pJEM15promo) even though a slight upward trend was observed in *M. smegmatis* mc^2155 *rpoB* H526Y (pJEM15promo) (Fig 5.3). However, the *M. tuberculosis* H37Rv *Rv1258c* promoter activity in *M. smegmatis* mc^2155 *rpoB* H526Y *attB*::pMV306promo*lacZ* was significantly different at approximately 1.5 times higher than that of *M. smegmatis* mc^2155 wild-type *attB*::pMV306promo*lacZ* (Fig 5.4).

5.3.3 *Rv1258c* promoter activity in response to rifampicin

The effect of RIF on *Rv1258c* promoter activity was investigated in *M. smegmatis*. To do this, the *M. smegmatis* S351L and H526Y *rpoB* mutants harbouring either the episomal or integrating vector systems were cultured to early log phase, split into two and exposed to RIF (10 μg/ml) or DMSO (diluent of RIF). The protein concentrations of WCLs were determined and β-galactosidase assays done. RIF did not affect *Rv1258c* promoter activity in either of the mutants transformed with the pJEM15promo*lacZ* (Fig 5.5) or pMV306promo*lacZ* (Fig 5.6) vector systems at one, three or six hours of RIF exposure. RIF also did not affect the background *Rv1258c* promoter activity, in other words the β-galactosidase activity in the strains transformed with the promoterless pJEM15 and pMV306*lacZ* vectors (results not shown).

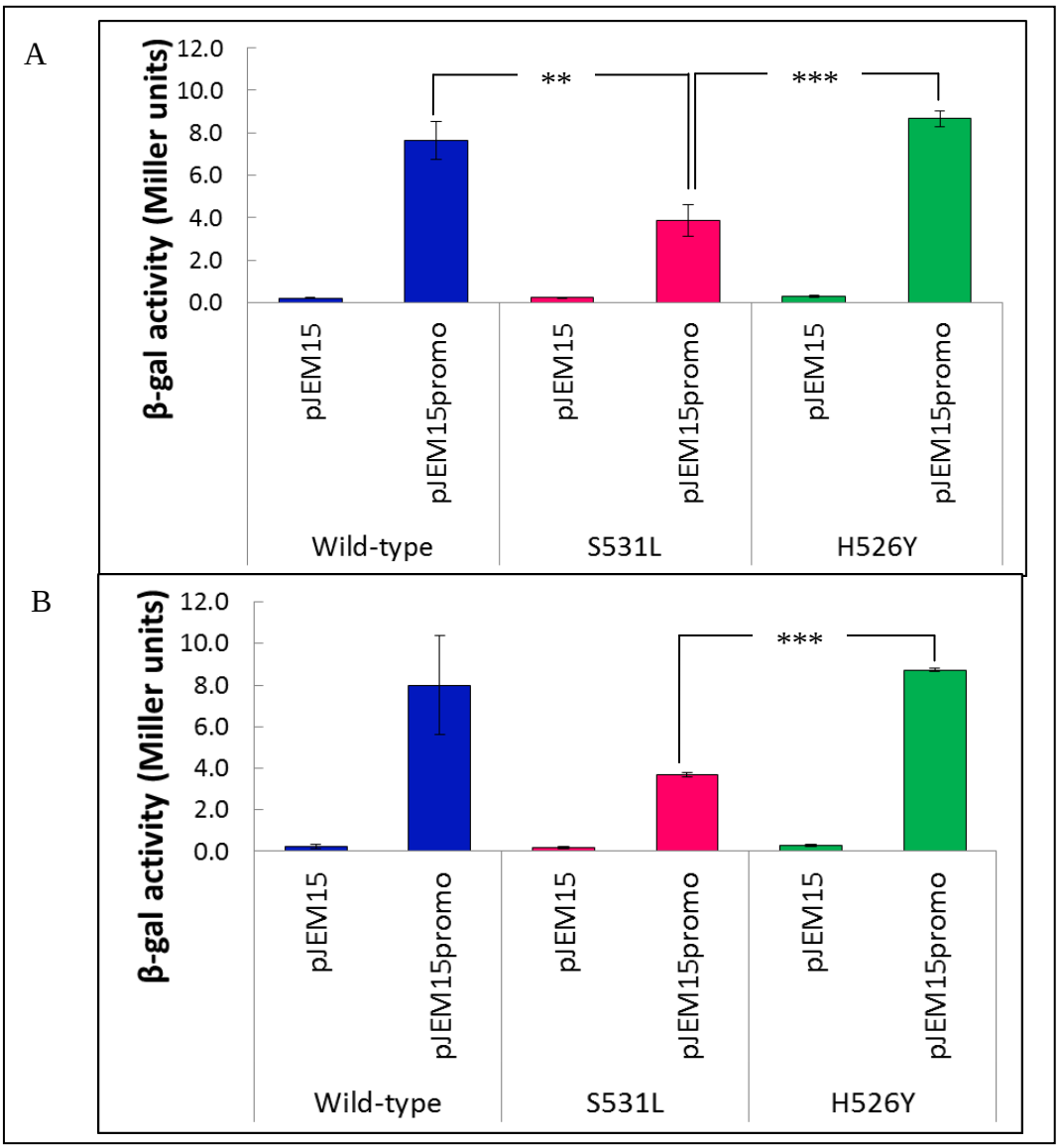


Figure 5.3. β-galactosidase activity for the *M. smegmatis* mc^{2}155 wild-type and S531L and H526Y *rpoB* mutants harbouring the episomal vectors at (A) early log phase and (B) three hours later. The value represents the average of three independent experiments and error bars represent standard deviation. Significant differences in *Rv1258c* promoter expression are indicated by $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

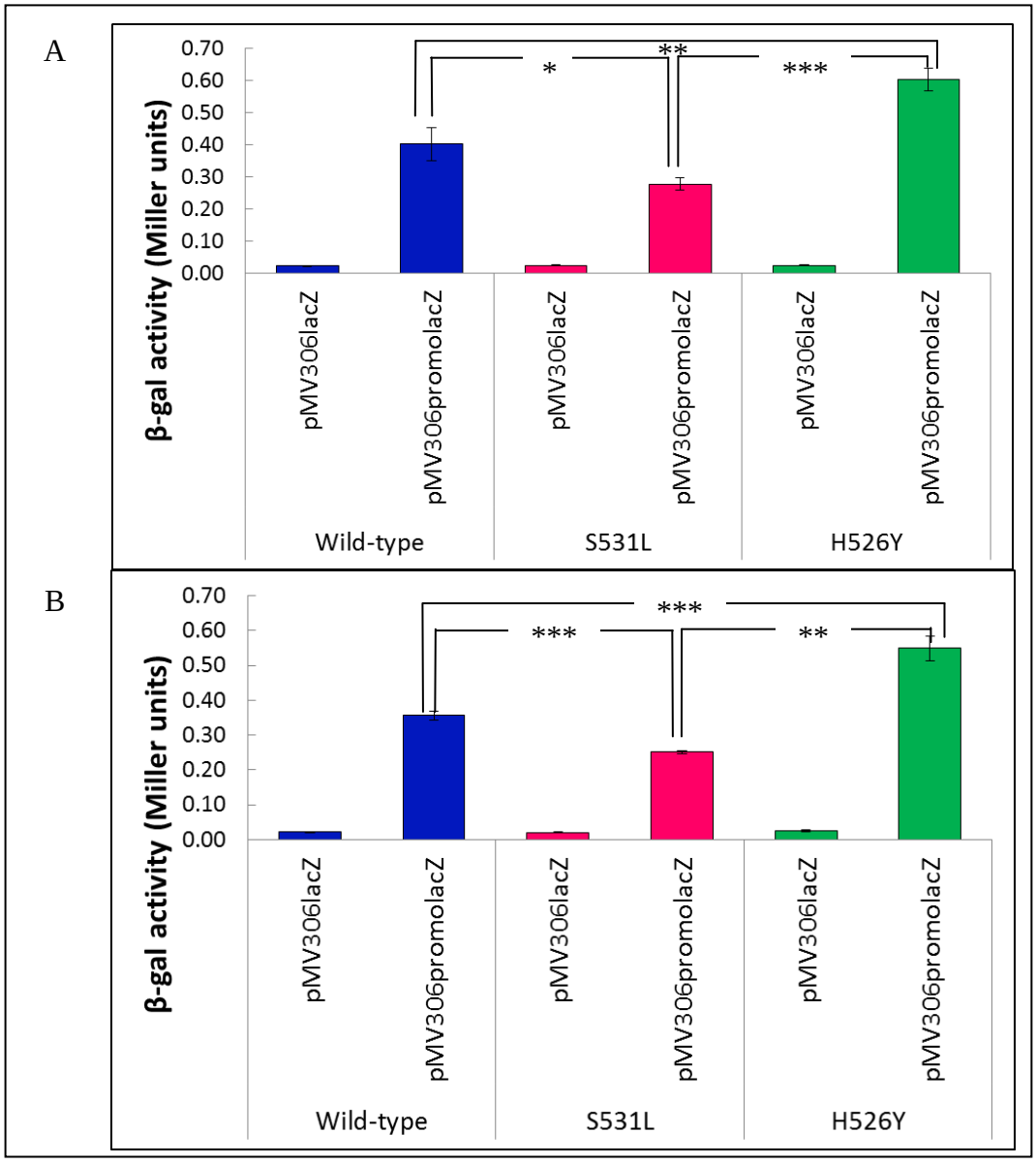


Figure 5.4. β-galactosidase activity for the *M. smegmatis* mc^{2}155 wild-type and S531L and H526Y *rpoB* mutants harbouring the integrating vectors at (A) early log phase and (B) three hours later. The value represents the average of three independent experiments and error bars represent standard deviation. Significant differences in *Rv1258c* promoter expression are indicated by $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

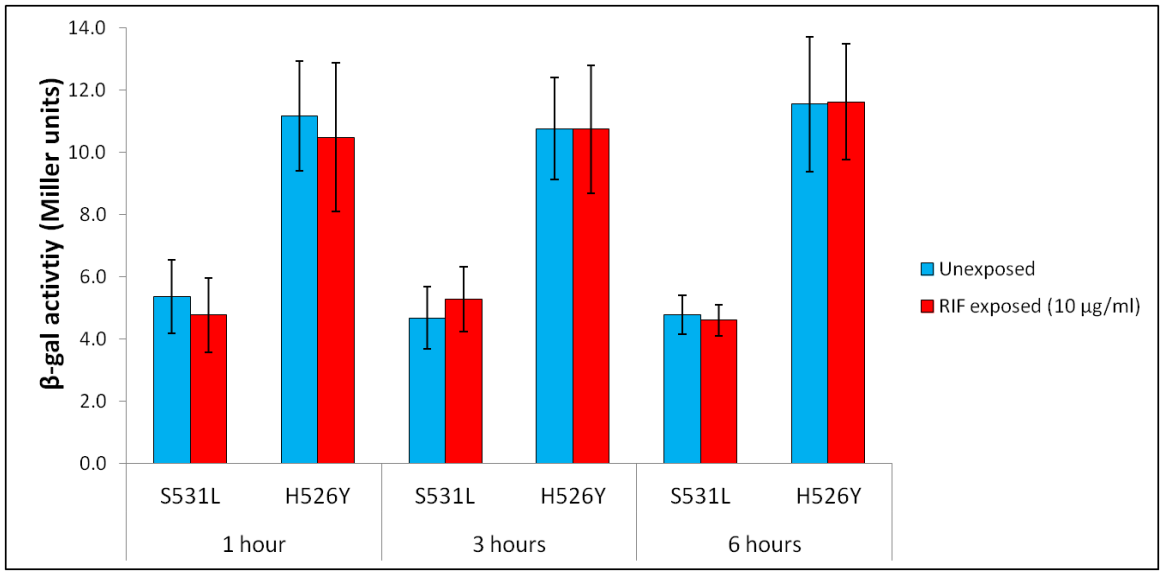


Figure 5.5. Effect of RIF on the *Rv1258c* promoter activity after 1, 3 and 6 hours of RIF (10 µg/ml) exposure for the *rpoB* mutants harbouring the pJEM15promo vector. The unexposed culture was exposed to DMSO (the diluent of RIF). The β-galactosidase values represent the average for three independent experiments and error bars represent standard deviation.

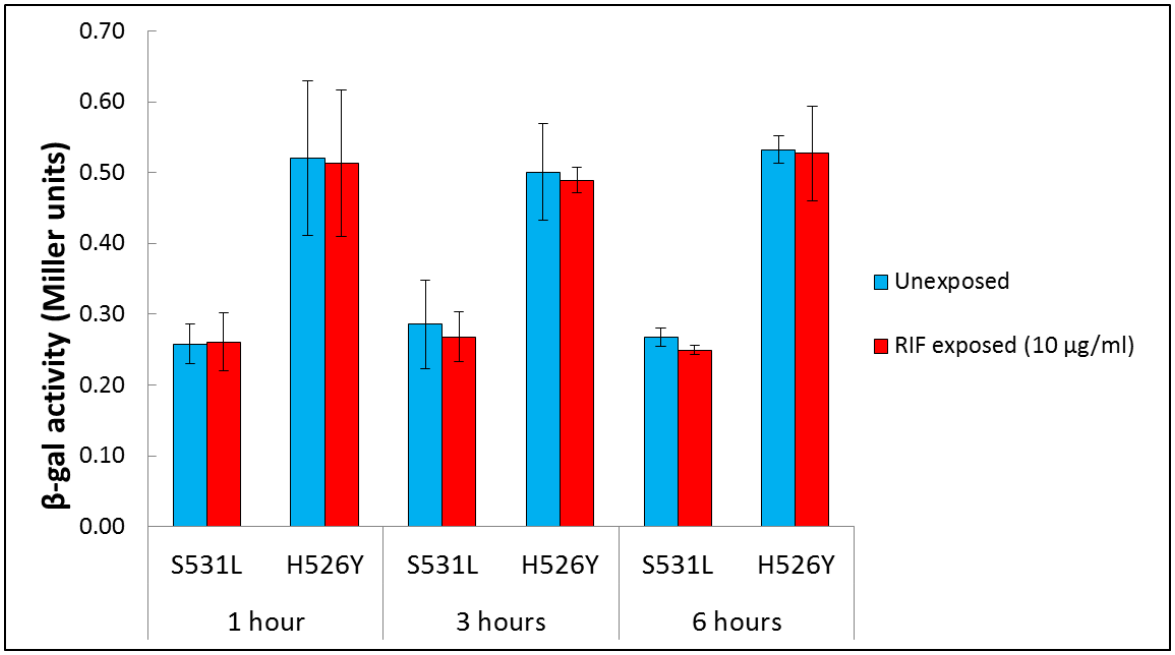


Figure 5.6. Effect of RIF on the *Rv1258c* promoter activity after 1, 3 and 6 hours of RIF (10 µg/ml) exposure for the *rpoB* mutants harbouring the pMV306promo*lacZ* vector. The unexposed culture was exposed to DMSO (the diluent of RIF). The β-galactosidase values represent the average for three independent experiments and the error bars represent standard deviation.

5.4 DISCUSSION

No difference was observed between the growth of the *M. smegmatis* mc^2155 strains transformed with the promoterless and promoter region containing vectors for either the episomal or integrating vector systems, indicating that expression of *lacZ* in these strains does not alter growth. The *rpoB* mutations affect the growth of the strains in comparison to the wild-type. The growth of the mutants was significantly slower than that of the wild-type during log phase. It has been shown before that the S531L and H526Y *rpoB* mutations lead to a slight fitness cost in *in vitro* generated *M. tuberculosis rpoB* mutants in comparison to the wild-type (Gagneux, 2009). The *rpoB* mutations did not have any extreme deleterious effect on the growth of the mutants and the mutants could still be cultured in a comparable number of hours to that of the wild-type.

Investigation of the *Rv1258c* promoter activity in the S531L and H526Y mutants revealed that the *M. tuberculosis* H37Rv promoter activity in the S531L mutant was significantly lower than that of the wild-type and this difference was observed for both the episomal (pJEM15promo) and integrating vector (pMV306promo*lacZ*) transformed strains. However there was only a slight upward trend in the *M. tuberculosis* H37Rv promoter activity of the H526Y mutant in comparison to that of the wild-type in the pJEM15promo transformed strains whereas there was a significant almost 1.5 fold higher promoter activity in the H526Y mutant in comparison to the wild-type in the strains transformed with pMV306promo*lacZ* vector. This suggests that having multiple copies of the *Rv1258c* promoter in the bacterium could influence the regulation of the *Rv1258c* promoter expression and we therefore chose to use the integrating vectors for the study in *M. tuberculosis* since there is only one copy of the vector per genome and this more accurately reflects the natural system. The integrating vector also has the advantage of being maintained more stably allowing the study to possibly be done in the absence of the selectable drug (Dennehy & Williamson, 2005).

When determining the effects of the *rpoB* mutations on *M. tuberculosis Rv1258c* promoter activity in *M. smegmatis*, the *rpoB* mutation affected *Rv1258c* promoter activity differently with a higher activity in the H526Y mutant and lower activity in the S531L mutant. Mutations in the *rpoB* gene have been shown to alter transcriptional pausing and termination (Yanofsky & Horn, 1981) and DNA supercoiling (Drlica *et al.*, 1988) and cause defects in transcription of the gene encoding the sigma subunit of RNA polymerase in *E. coli* (Zhou &

Jin, 1997) and in the process alter gene expression. In *B. subtilis*, each *rpoB* mutation differentially affected gene expression and had their own unique effect on growth rate in various media, substrate utilization patterns, the expression of competence for transformation, sporulation frequency, sporulation efficiency, spore resistance to heat and chemicals and germination (Maughan *et al.*, 2004; Perkins & Nicholson, 2008; Moeller *et al.*, 2012). Mutations in *rpoB* also affect antibiotic production in *S. lividans* and *S. coelicolor* (Hu & Ochi, 2001; Hu *et al*., 2002). Some of these effects of *rpoB* mutations might also be present in other bacteria including *M. smegmatis*, together with other unknown effects since the effects of *rpoB* mutations on mycobacterial gene expression has not been studied extensively. Structural changes in RNA polymerase might occur as a result of *rpoB* mutations which may influence the binding to or interaction of RNA polymerase with transcription factors resulting in altered expression of *Rv1258c*. Differences in *Rv1258c* promoter activity were therefore not unexpected since gene expression could be influenced by *rpoB* mutations.

Investigation of the effects of RIF (10 µg/ml) on *Rv1258c* promoter activity in *M. smegmatis* using the reporter systems developed, revealed that RIF did not affect the promoter activity. The induction of *Rv1258c* in only some MDR clinical isolates and a H37Rv RIF mono-resistant mutant of *M. tuberculosis*, but not in others was observed following RIF exposure (Siddiqi *et al.*, 2004; Gupta *et al.*, 2006; Jiang *et al.*, 2008; Louw *et al.*, 2011). The lack of induction in this study may be due to several reasons. Firstly, induction upon RIF exposure might depend on the strain used and therefore be a phenotype of that specific strain as suggested by the aforementioned studies in *M. tuberculosis.* Secondly, the concentration of the RIF used (10 µg/ml) might not have been high enough to induce *Rv1258c* efflux pump expression in the *rpoB* mutants with such a high RIF MIC (125 -200 µg/ml). A RIF concentration of 10 µg/ml was chosen since it was higher than the RIF MIC for the wild-type strain, but this may not be sufficient. *M. smegmatis* has intrinsic resistance to RIF and two mechanisms have been implicated in this resistance. One mechanism is the inactivation of RIF by rybosylation that occurs in *M. smegmatis*, but not *M. tuberculosis*. Cloning of the gene that is responsible for rybosylation into a related organism, conferred low-level RIF resistance and disruption of the gene in *M. smegmatis* increased susceptibility to RIF. This shows that rybosylation contributes to the RIF resistance of *M. smegmatis* (Quan *et al.*, 1997).

MsRbpA is a protein that is present in *M. smegmatis* and has homologues in other actinomycetes including *M. tuberculosis* (RbpA), *M. leprae* and *Streptomyces coelicolor* (Newell *et al.*, 2006; Dey *et al.*, 2010). This protein was shown to interact with and bind to the RNA polymerase in *M. smegmatis* and *S. coelicolor*. It was suggested that the MsRbpA protein (from *M. smegmatis*) and RbpA (from *S. coelicolor*) might protect or modify the RNA polymerase thereby preventing the effects of RIF (Newell *et al.*, 2006; Dey *et al.*, 2010). Overexpression of MsRbpA in *M. smegmatis* increased RIF resistance in otherwise RIF sensitive strains, while deletion of the *S. coelicolor* homologue *rbpA*, increased its sensitivity to RIF and *rbpA* was essential for growth. The MsRbpA protein and its homologue RbpA is thus involved in low-level RIF resistance in *M. smegmatis* and *S. coelicolor*. *M. tuberculosis* RbpA was suggested to act as a transcriptional activator by affecting the RNA polymerase structure and in the process increasing its affinity for the sigma factor A thereby facilitating transcription even in the presence of RIF. RbpA thus indirectly contributes to the RIF resistance in *M. tuberculosis* as well (Hu *et al.*, 2012). The intrinsic resistance of *M. smegmatis* may therefore mean that a much higher RIF concentration is required to induce *Rv1258c* expression.

Thirdly, although *M. smegmatis* is a useful model organism its utility may be limited due to differences between this fast-growing non-pathogenic organism and *M. tuberculosis.* Since the *Rv1258c* homologue in *M. smegmatis*, *MSMEG_5033*, is a pseudogene the regulation of *Rv1258c* may not be conserved in this organism and therefore the expression of the *Rv1258c* promoter in *M. smegmatis* might not be representative of expression of the *Rv1258c* promoter in *M. tuberculosis*. In addition, the slight differences in the RNA polymerase of *M. smegmatis* may alter its effect on the *M. tuberculosis* promoter. In conclusion, while the analysis of the *M. tuberculosis Rv1258c* promoter activity in *M. smegmatis* was useful to evaluate the functionality of the episomal and integrating vectors for the subsequent promoter reporter studies in *M. tuberculosis* and providing an idea of what could be expected in *M. tuberculosis*, it might not represent what happens in *M. tuberculosis*.

CHAPTER 6: EFFECT OF *RPOB* MUTATIONS ON *RV1258C* PROMOTER ACTIVITY IN *M. TUBERCULOSIS*

6.1 INTRODUCTION

RNA polymerase can regulate gene expression not only at the gene's promoter operator site, but also at their transcriptional termination site or attenuator. Mutations in *rpoB* have been shown to affect transcriptional termination negatively (Yanofsky & Horn, 1981). Expression of the tryptophan (*trp*) operon in *E. coli* in *rpoB* mutants was altered because the mutations somehow influenced RpoB's recognition of the transcriptional terminator and caused either increased or decreased expression of *trp* (Yanofsky & Horn, 1981). Mutations in *rpoB*, causing amino acid substitutions not associated with RIF resistance in *E. coli* was also shown to alter transcriptional pausing and termination (Landick *et al.*, 1990). This indicates how *rpoB* mutations affect the expression of genes through its effect of transcriptional termination.

Mutations in *rpoB* do not only affect transcription by influencing transcriptional termination, but also by altering DNA supercoiling (Drlica *et al.*, 1988). In bacteria, DNA supercoiling is controlled by DNA gyrase, causing negative supercoils, and DNA topoisomerase, which modulates negative supercoiling by gyrase. DNA supercoiling affects transcription. Mutations in *gyrB* cause DNA relaxation. Mutations in the *rpoB* gene caused a reduced DNA relaxation in the *gyrB* mutant, indicating how RNA polymerase can alter DNA supercoiling and thus possibly also transcription (Drlica *et al.*, 1988).

Mutations in *rpoB* caused defective transcription of the gene encoding the sigma subunit of RNA polymerase in *E. coli* (Zhou & Jin, 1997). Since sigma factors bind to the core of RNA polymerase consisting of three subunits including the β-subunit, and this complex then recognizes and binds to specific promoter, transcription of other genes would presumably also be influenced by defective transcription of the gene encoding the sigma factor and thus by *rpoB* mutations (Zhou & Jin, 1997).

Similarly to *M. tuberculosis*, numerous mutations in *rpoB* have been identified which lead to RIF resitance in *B. subtilis* spores as well as vegetative cells. The S487L mutation corresponding to the S531L mutation in *M. tuberculosis* is also the predominant *rpoB*

mutation in *B. subtilis* spores (Nicholson & Maughan, 2002). Besides from the RIF resistance phenotype associated with the *rpoB* mutations in *B. subtilis*, each *rpoB* mutation differentially affected gene expression and had their own unique effect on growth rate in various media, substrate utilization patterns, the expression of competence for transformation, sporulation frequency, sporulation efficiency, spore resistance to heat and chemicals and germination (Maughan *et al*., 2004; Perkins & Nicholson, 2008; Moeller *et al.*, 2012).

In *S. lividans*, *rpoB* mutations are also associated with RIF resistance. *S. lividans* produces antibiotics including actinorhodin (Act), undecylprodigiosin (Red), methylenomycin and calcium-dependent antibiotic (CDA). Act, Red and CDA biosynthesis are induced in the presence of *rpoB* mutations depending on the position of the *rpoB* mutation and the amino acid substitution caused (Hu *et al*., 2002). Mutations in *rpoB* enhanced Act production in *S. coelicolor*. Interestingly, the presence of other mutations conferring streptomycin and gentamicin resistance respectively also increase the production of Act and a combination of mutations conferring resistance to all three drugs have an additive effect on Act production (Hu & Ochi, 2001). The *rpoB* mutations thus affect transcription of numerous genes involved in many different processes.

It is tempting to speculate that the effects of *rpoB* mutations are not limited to those discussed, but are also felt at many other levels in bacteria, as it forms part of RNA polymerase that is responsible for transcription and gene regulation. In line with this argument, *rpoB* mutations in *M. tuberculosis* are generally associated with a fitness cost under certain conditions, depending on the *rpoB* mutation and strain (Mariam *et al.*, 2004; Gagneux, 2009). However, the S531L and H526Y mutations were associated with a low fitness cost in *in vitro* generated *rpoB* mutants under the conditions tested (Gagneux, 2009).

RIF binds to RpoB and forms a stable drug-enzyme complex and directly blocks the RNA transcript elongation when the transcript is just two to three nucleotides long, thereby inhibiting gene transcription (Campbell *et al.*, 2001; Johnson *et al.*, 2006). The effects of RIF on *Rv1258c* expression in some strains lead us to hypothesise that the *rpoB* mutations present affects the expression of *Rv1258c* and this *Rv1258c* expression is altered by RIF exposure.

6.2 METHODOLOGY

6.2.1 Selection of spontaneous *M. tuberculosis* H37Rv *rpoB* mutants

M. tuberculosis H37Rv spontaneous *in vitro rpoB* mutants were selected as described by (Morlock *et al.*, 2000), which is an adaptation of the fluctuation test originally designed by Luria & Delbrück (1943). According to the Morlock *et al.* (2000) method, a *M. tuberculosis* H37Rv starter culture was cultured to an OD of approximately 0.80 and diluted 100 000 times in 7H9 supplemented with 10% OADC (Sigma-Aldrich) (7H9 OADC) to approximately 10^3 colony forming units (CFU)/ml. It was pre-determined that the concentration of the culture would be approximately 10^8 CFU/ml at a culture OD of 0.80 at 600 nm. Aliquots (5 ml) of this low density culture was aliquoted into 100 flasks. These cultures were incubated at 37°C for 28 days with daily shaking, allowing the cultures to reach a high density in order to obtain spontaneous RIF resistance mutations. The 5 ml cultures were then transferred into 50 ml conical tubes and pelleted by centrifugation at 3,220 ×g for 3 min. The supernatant was discarded and the pellet was re-suspended in 0.5% Tween 80. After centrifugation at 3,220 ×g for 3 min at room temperature, the pellet resuspended in 1.0 ml of the supernatant. A 100 µl aliquot was then plated onto 7H10 supplemented with 10% OADC containing 2 µg/ml RIF in order to select for RIF resistant mutants. After four weeks incubation at 37°C, two colonies were picked from each plate and inoculated into 7H9 ADC. Stocks were prepared for each of the 200 cultures and an aliquot of each culture (200 µl) was boiled to be used for PCR.

A 437 bp region containing the RRDR was amplified using the rpoBFor and rpoBRev primers (Table 3.4). The HotStarTaq DNA Polymerase kit (Qiagen) was used. Each 25 µl reaction contained 1x PCR buffer, 400 µM dNTPs, 1 mM $MgCl_2$, 0.5 µM rpoFor and rpoBRev, 0.75 U HotStarTaq DNA Polymerase and 2 µl of boiled culture. The run protocol was as follows: initial denaturation and enzyme activation at 95°C, followed by three sets of 2 cycles each of denaturing at 94°C, elongation at 72°C, 71°C and 70°C respectively and annealing at 72°C, followed by 40 cycles of denaturation at 94 °C, annealing at 69°C and elongation at 72°C, followed by a final 10 min elongation at 72°C. The PCR product (5 µl) was loaded onto a gel to determine whether amplification was successful. The PCR product was sequenced by the Central Analytical Facility at Stellenbosch University using the

rpoBFor primer in order to determine whether mutations occurred within the *rpoB* gene and if so, which mutations.

6.2.2 Determining the MIC for the *rpoB* mutants

The MIC for the *M. tuberculosis* H37Rv *rpoB* mutants was determined using the automated BACTEC MGIT 960 instrument (BD Biosciences) according to the manufacturer's instructions. *M. tuberculosis* freezer stocks (200 µl) were inoculated into MGIT tubes supplemented with 10% OADC. The culture was incubated in the MGIT instrument at 37°C until a positive growth reading was reached, then incubated for a further two days at 37°C. A 1:100 dilution of the culture in sterile saline was prepared and 500 µl of this dilution was inoculated into a new MGIT tube. This MGIT culture would serve as the growth control. The undiluted MGIT culture (500 µl) was inoculated into MGIT tubes containing a range of RIF concentrations (50 – 200 µg/ml). The MGIT cultures were placed in a BACTEC MGIT 960 instrument and EpiCentre (version 5.75A) TBeXist software (BD Bioscience) was used to monitor the culture growth for approximately 14 days and to analyse the data generated by the MGIT 960 instrument.

6.2.3 β-galactosidase assays to determine loss of integrating vector

M. tuberculosis H37Rv wild-type and *rpoB* mutant strains were transformed with the integrating vector systems. Pre-cultures of *M. tuberculosis* H37Rv wild-type and *rpoB* mutant strains transformed with the integrating vector systems were cultured in 7H9 ADC containing the selectable antibiotic, hygromycin. The pre-cultures were sub-cultured into 7H9 ADC containing no hygromycin to a starting OD of 0.05 at 600 nm. WCLs were prepared as previously described at early log growth phase and after further one and seven days. The protein concentrations of the WCLs were determined and β-galactosidase assays done as previously described.

6.2.4 Growth curve analysis of transformed *M. tuberculosis*

Growth curves were generated for all the *M. tuberculosis* H37Rv wild-type and mutant strains harbouring the integrating vectors. Pre-cultures were cultured in 7H9 ADC containing the appropriate selectable drug, hygromycin. The pre-cultures were sub-cultured into 80 ml

7H9 ADC not containing any selectable drug to an OD of 0.05 at 600 nm. The OD at 600 nm was measured every day for 14 days using a Novaspec II visible spectrophotometer (LKB). The same *M. tuberculosis* cultures that were used for the β-galactosidase non-exposure experiments were used for the growth curves (in other words, the culture volume gradually decreased as volumes of the culture were removed for WCL preparation and RNA extraction).

6.2.5 Promoter activity in the absence of rifampicin

The effect of *rpoB* mutations on *Rv1258c* expression was determined by comparing *Rv1258c* expression in *M. tuberculosis* H37Rv wild-type *attB*::pMV306promo*lacZ*, *M. tuberculosis* H37Rv *rpoB* S531L *attB*::pMV306promo*lacZ* and *M. tuberculosis* H37Rv *rpoB* H526Y *attB*::pMV306promo*lacZ*. The background expression level of *lacZ* was also determined in the strains transformed with the pMV306*lacZ* vector. Pre-cultures were cultured in 7H9 ADC containing the appropriate selectable drug, hygromycin. The pre-cultures were sub-cultured into 80 ml 7H9 ADC not containing any selectable drug to an OD of 0.05 at 600 nm. At an OD between 0.6 to 0.75 WCLs were prepared (0 hours) as well as 24 hours later (24 hours) later. The protein concentrations of the WCLs were determined and β-galactosidase assays done.

6.2.6 Promoter activity in response to rifampicin

Each pre-culture of *M. tuberculosis* H37Rv S531L and H526Y *rpoB* mutants harbouring the integrating vectors were sub-cultured into two separate 80 ml cultures. After culturing to early log-phase, one of the two cultures for each strain was exposed to RIF (2 μg/ml) while the other culture was exposed to DMSO (diluent of RIF). Exposure to DMSO was done to determine the RIF-specific response. The secondary container for the RIF exposed cultures was covered with tinfoil in order to prevent any light from entering since RIF is light sensitive. WCLs were prepared for both the exposed and unexposed cultures after 1, 2 and 24 hours of RIF exposure. The protein concentrations of the WCLs were determined and *Rv1258c* promoter activity examined using β-galactosidase assays.

6.3 RESULTS

The effect of *rpoB* mutations on *Rv1258c* expression was investigated using β-galactosidase assays in *M. tuberculosis rpoB* mutants in the absence and presence of RIF.

6.3.1 Selection of spontaneous *M. tuberculosis* H37Rv *rpoB* mutants

M. tuberculosis H37Rv spontaneous *in vitro rpoB* mutants were selected in order to compare the influence of *rpoB* mutations on *Rv1258c* expression in mutants which presumably only have a SNP in *rpoB*. A range of *rpoB* mutations were present among the 94 mutant colonies picked and the mutations as well as their distribution are indicated in Fig 6.1. The mutation that occurred most frequently are the H526Y (27.7%) mutation followed by the H526R (23.4%) and H526D (17%) mutations. S531L mutants occurred at a frequency of 9.6%.

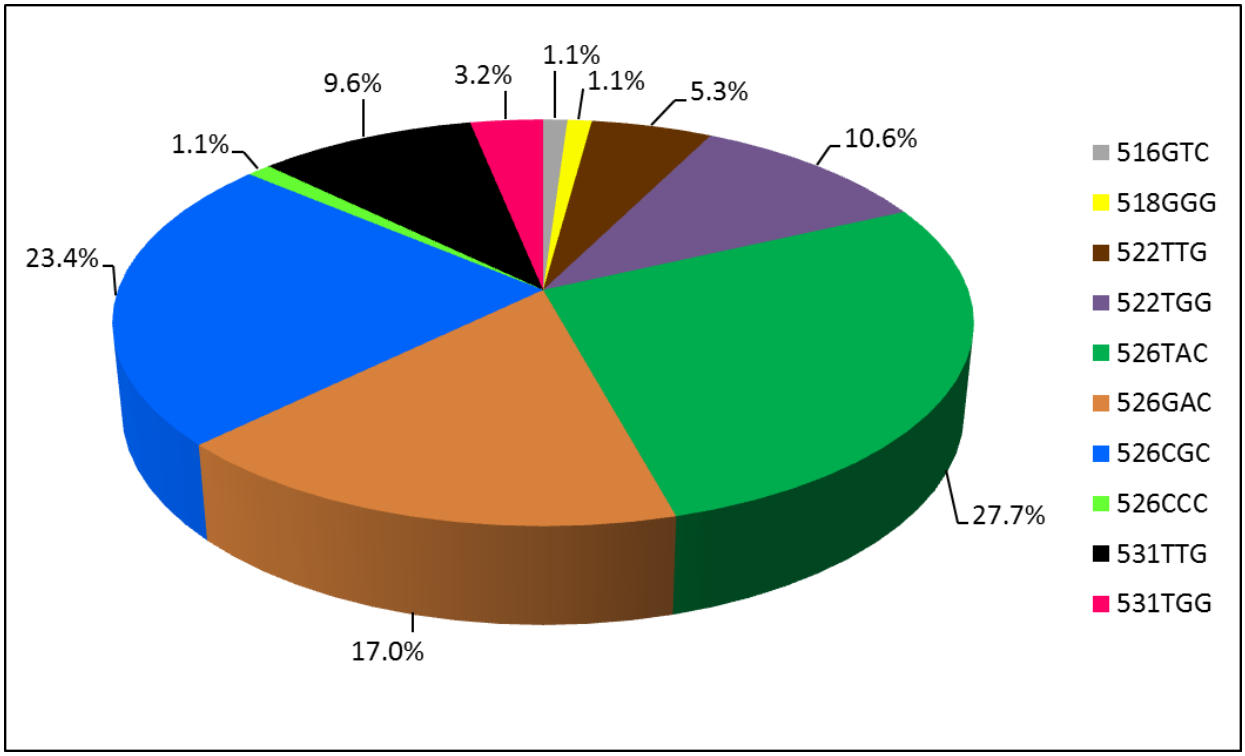


Figure 6.1. The frequency of spontaneous *in vitro* generated *rpoB* mutants.

6.3.2 Determining the MIC for the *rpoB* mutants

A S531L and H526Y *rpoB* mutant was chosen from the library of mutants generated. The RIF MIC for these mutants were determined in order to correlate results observed for

subsequent studies with the level of RIF resistance observed for the mutants. The growth units (GU) of the RIF exposed MGIT cultures were compared to that of the growth control. At the time at which the growth control reached a GU of above 400, the GU of the drug containing tubes were compared and if the latter was more than or equal to 100, the strain would be considered resistant at that specific drug concentration. If the GU at a drug concentration was less than 100, incubation was prolonged for another 7 days, until the growth control reaches a GU of more than 400. If the GU was still less than 100 after 7 days, the strain was considered as sensitive to that drug concentration. If the GU increased to 400 within 7 days, the strain was considered intermediately resistant at the specific drug concentration.

The growth control for the S531L mutant only started to come up at 12 days and 22 hours, and had not reached a GU of above 400 yet at this time point (Fig 6.2). The GU of the drug containing cultures were however still compared to that of the growth control at this stage, since it is not advisable to go beyond 14 days when examining the MIC using this system. At this point, the GU for the 50 µg/ml and 100 µg/ml RIF exposed cultures were above 400, indicating that the S531L mutant was resistant at these RIF concentrations. After 12 days and 22 hours of exposure, the GU of the 150 µg/ml RIF exposed culture started to increase (Fig 6.2). The S531L mutant was thus resistant to 100 µg/ml RIF, and intermediately resistant to 150 µg/ml and sensitive to 200 µg/ml RIF. The MIC for S531L is thus between 150 µg/ml

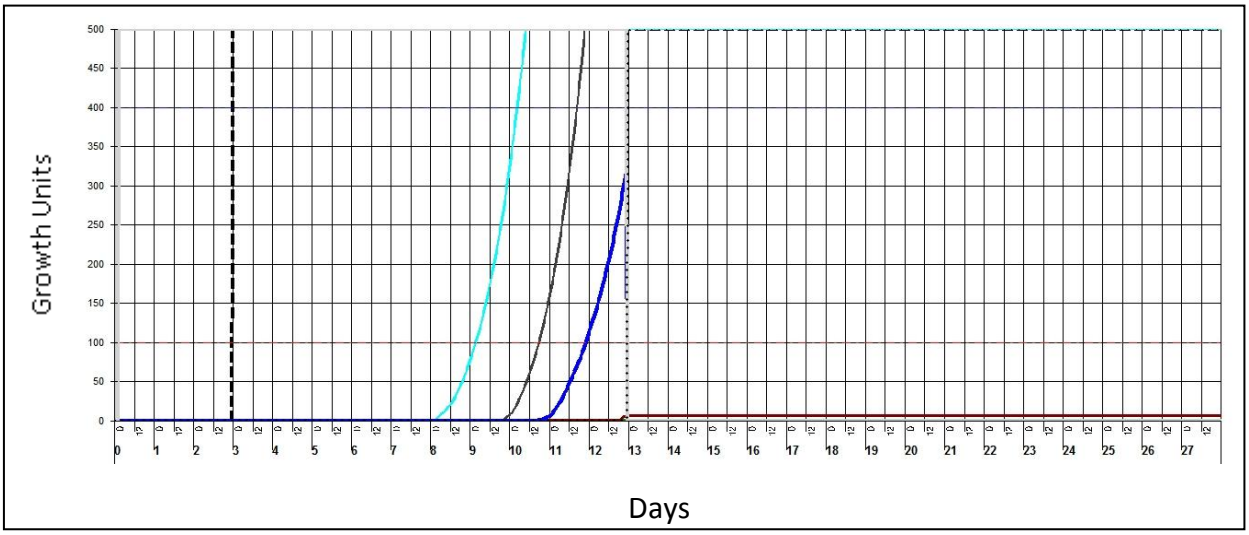


Figure 6.2. MGIT 960 eXIST Plot of GU measured over days for the S531L mutant. The light blue line represents 50 µg/ml RIF, dark grey line represents 100 µg/ml, maroon line represents 150 µg/ml and the dark blue line represents the growth control.

and 200 µg/ml. From Fig 6.3 it can be seen that the GU was more than 400 for the 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml H526Y RIF exposed culture at the time when the growth control reached a GU above 400. This means that the H526Y mutant was resistant to all these RIF concentrations and has a MIC of equal to or above 200 µg/ml.

6.3.3 β-galactosidase assays to determine loss of integrating vector

Since integrating vectors systems are more stable than episomal vectors, the vector may be maintained in the absence of the selectable antibiotic, hygromycin. In order to assess the stability of the integrating vector in the absence and presence of selectable antibiotic,

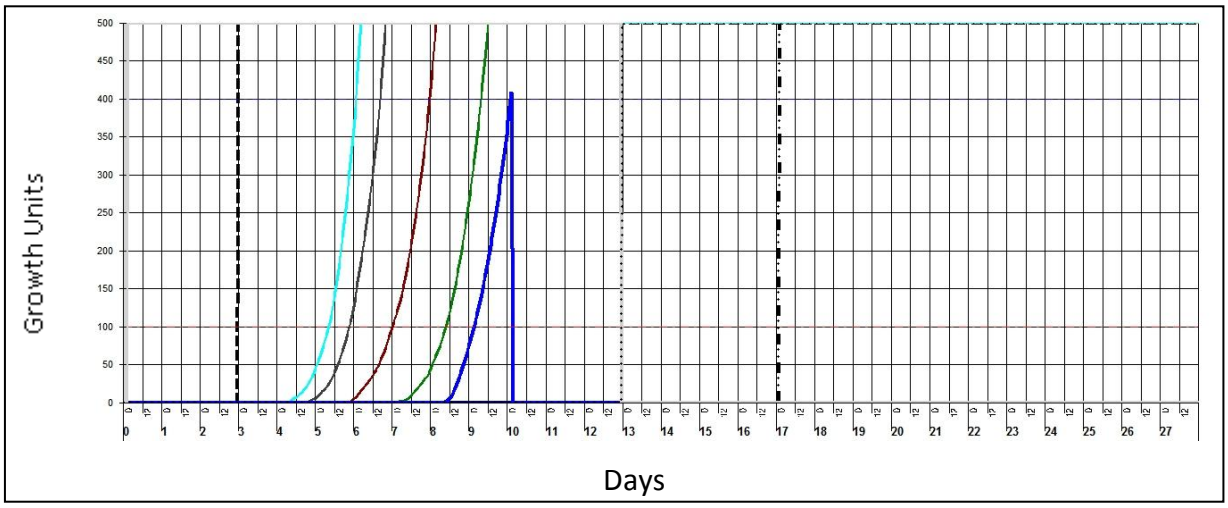


Figure 6.3. MGIT 960 eXIST Plot of GU measured over days for the H526Y mutant. The light blue line represents 50 µg/ml RIF, dark grey line represents 100 µg/ml, maroon line represents 150 µg/ml, green line represents 200 µg/ml and the dark blue line represents the growth control.

Rv1258c promoter activity was compared between *M. tuberculosis* H37Rv *attB*::pMV306promo*lacZ* cultured in the absence and presence of hygromycin. WCLs were prepared and β-galactosidase assays done in order to monitor the loss of the integrating vector when culturing in the absence of hygromycin. *Rv1258c* promoter expression levels were monitored using β-galactosidase assays and served as an indicator of the presence of the integrating vector and thus of the loss of integrating vector. The *Rv1258c* promoter expression levels were comparable in the cultures exposed to hygromycin and those that were cultured in the absence of hygromycin at early log phase (day 0) as well as 1 day after this

(Fig 6.4). There was only a slightly lower expression of *Rv1258c* promoter in the absence as compared to in the presence of hygromycin after 7 days of culturing (Fig 6.4).

However, only one experiment was done. The results obtained for this experiment suggested that there was no loss of integrating vector when culturing was done in the absence of the selectable antibiotic, hygromycin, on day 0 and 1. The culturing of *M. tuberculosis* transformed with the integrating vector was subsequently done in the presence of hygromycin for the pre-culture and upon sub-culturing in the absence of hygromycin.

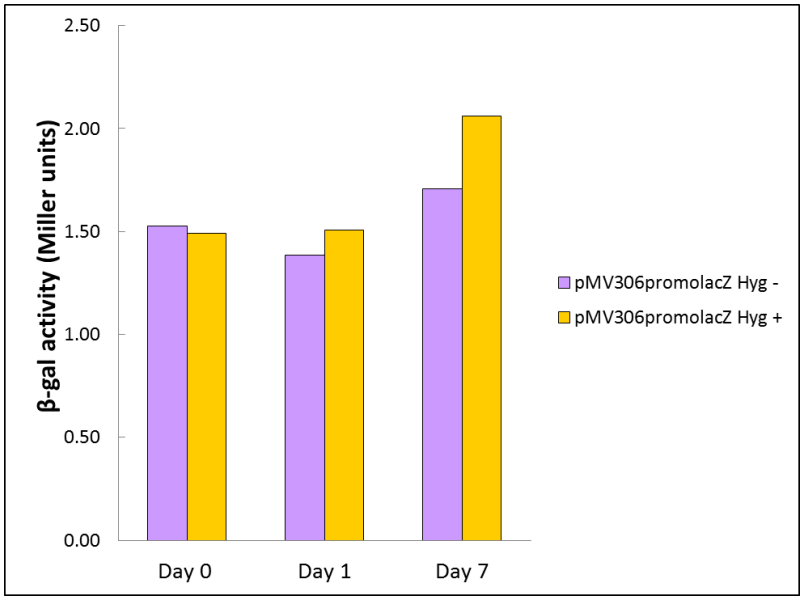


Figure 6.4. Comparison of *Rv1258c* promoter activity in the absence and presence of hygromycin, to monitor the loss of the pMV306promo*lacZ* vector. Day 0 represents the OD of between 0.60 and 0.70 (early log phase) at which point WCLs for β-galactosidase assays would be prepared. Day 1 and Day 7 represent 1 and 7 days thereafter. The β-galactosidase values represent only one experiment.

6.3.4 Growth curve analysis of transformed *M. tuberculosis*

Growth curves were generated for the *M. tuberculosis* wild-type and *rpoB* mutants harbouring the integrating vectors. This was done in order to determine if the presence of the vectors influenced the growth of the bacteria, the effects of the *rpoB* mutations on the growth of the *M. tuberculosis rpoB* mutant and the OD at which the log growth phase was reached (since the WCLs for the β-galactosidase assays would be prepared at early log phase). No significant differences in growth of the wild-type and *rpoB* mutants were observed (Fig 6.5)

although the H526Y mutant tended to grow slower than the wild-type and S531L mutant tended to grow faster than the wild-type during log growth phase. The cultures entered log phase at an OD of approximately 0.60 to 0.75.

6.3.5 Promoter activity in the absence of rifampicin

The effect of *rpoB* mutations on *Rv1258c* expression was determined by comparing *Rv1258c* promoter activity in *M. tuberculosis* H37Rv wild-type and S531L and H526Y *rpoB* mutants harbouring the integrating vectors at early log growth phase as well as 24 hours thereafter.

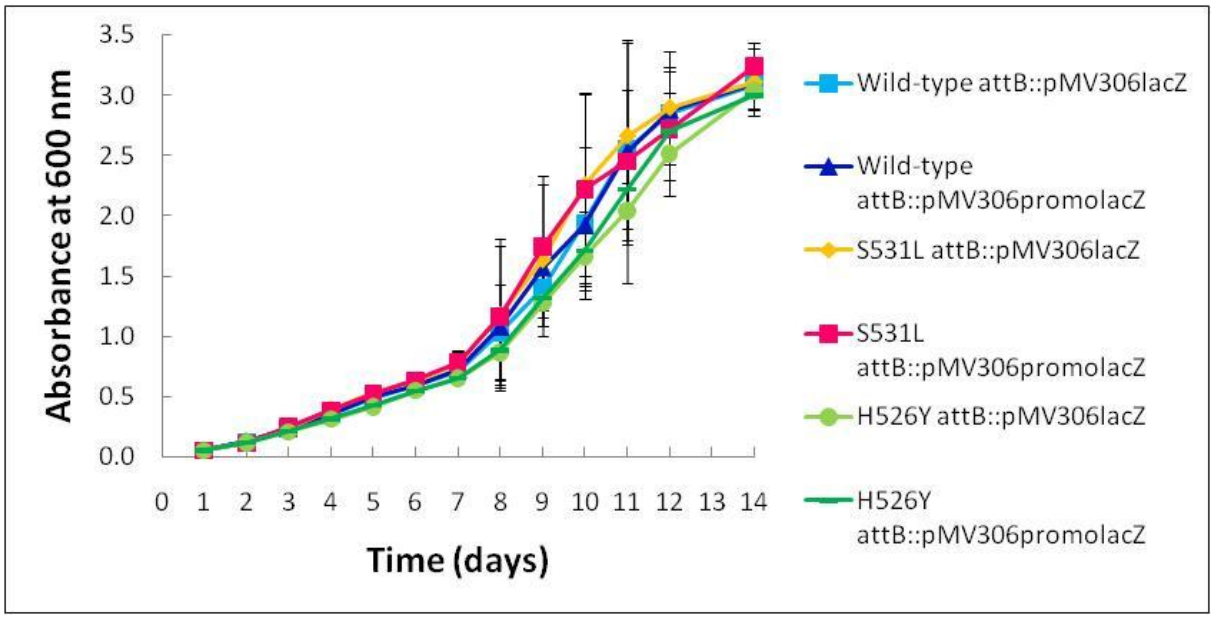


Figure 6.5. Growth curves for *M. tuberculosis* wild-type and *rpoB* mutants harbouring the integrating vectors. Pre-cultures were cultured in the presence of hygromycin and hygromycin was omitted upon sub-culturing. The values represent the average of three independent experiments and error bars represent standard deviation.

Rv1258c promoter activity of the strains transformed with pMV306*lacZ* indicates the background activity in the absence of a promoter. This background activity was negligible (Fig 6.6) as expected since there is a transcriptional terminator upstream of the *lacZ* gene in the pMV306*lacZ* vector. *Rv1258c* promoter activity in the integrating vector transformed S531L mutant was significantly lower at approximately half of that of the wild-type, while *Rv1258c* promoter activity in the H526Y mutant was significantly higher at approximately double that of wild-type. There was an approximately four fold difference in *Rv1258c* promoter activity between the S531L and the H526Y mutant. The promoter activity for the

early log phase culture (0 hours) (at culture OD of between 0.6 and 0.75) and 24 hours thereafter, were comparable (Fig 6.6).

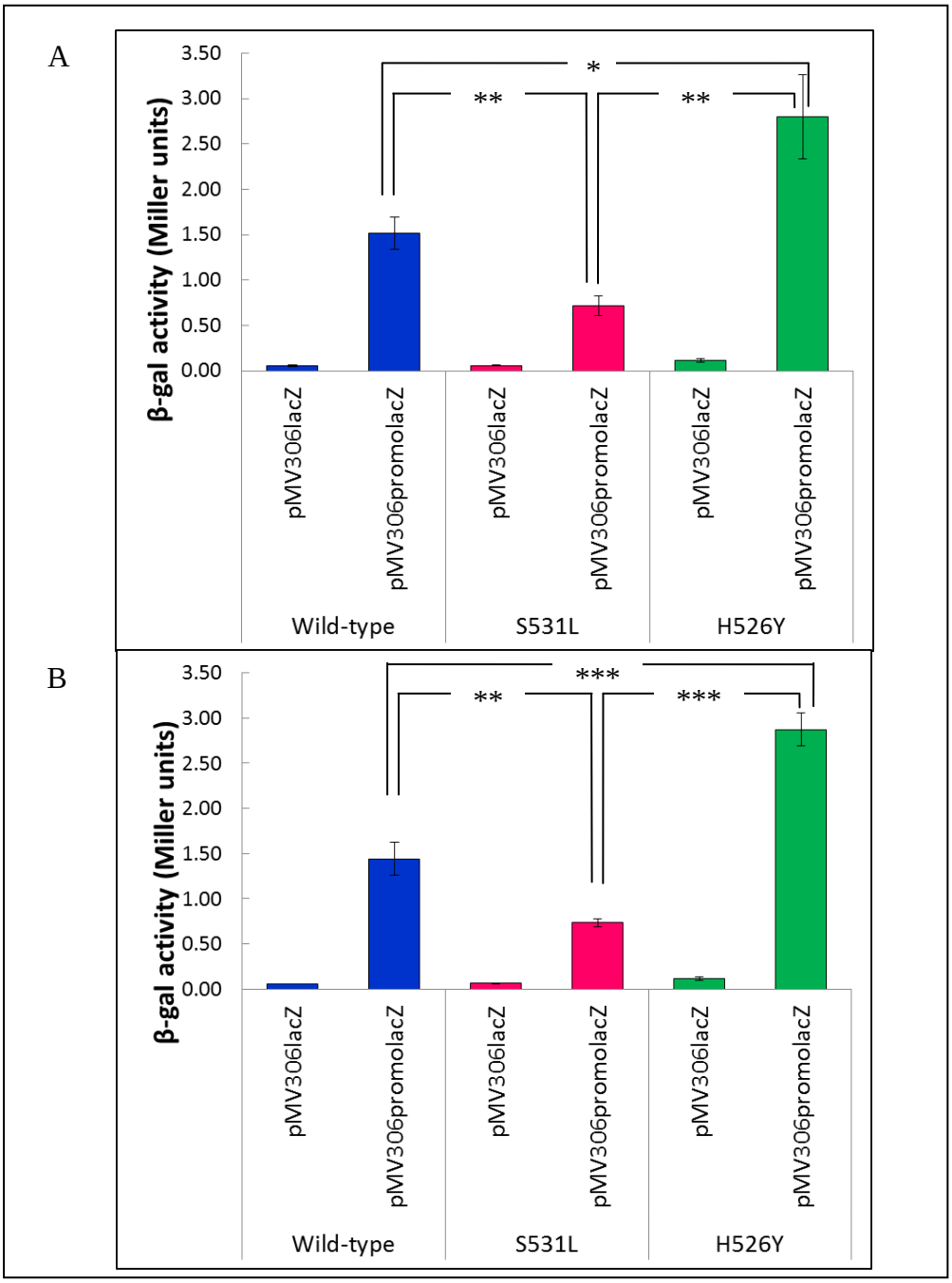


Figure 6.6. β-galactosidase activity of the *M. tuberculosis* H37Rv wild-type and S531L and H526Y *rpoB* mutants harbouring integrating vectors at (A) early log phase (0 hours) and (B) 24 hours thereafter (24 hours). The β-galactosidase value represents the average of three independent experiments and error bars represent standard deviation. Significant differences in *Rv1258c* promoter expression are indicated by $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

6.3.6 Promoter activity in response to rifampicin

In order to determine the effect of RIF on *Rv1258c* expression, *M. tuberculosis* S531L and H526Y *rpoB* mutants harbouring the integrating vectors were cultured to early log phase and exposed to 2 µg/ml RIF. WCLs were prepared after 1, 2 and 24 hours of RIF exposure and *Rv1258c* expression examined using β-galactosidase assays. RIF did not affect *Rv1258c* promoter activity in the either of the *rpoB* mutants at 1, 2 or 24 hours of RIF exposure. As expected, RIF did not affect the expression of the promoterless integrating vector, pMV306*lacZ* at 1, 2 or 24 hours of exposure (results not shown).

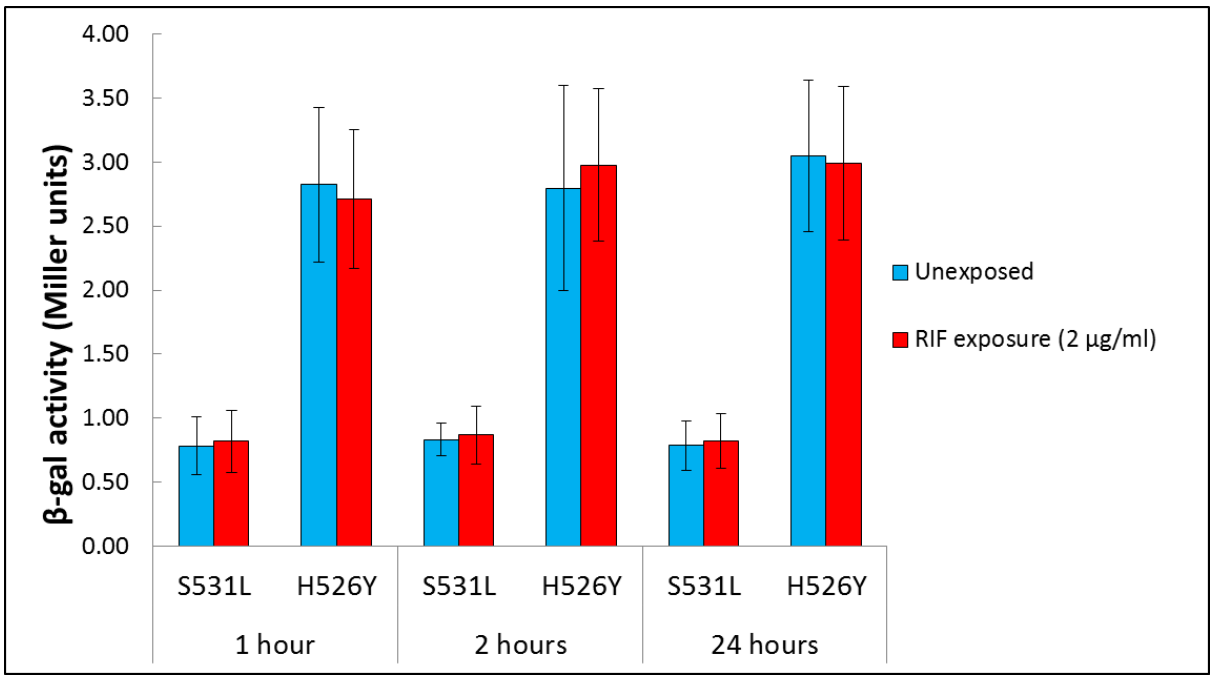


Figure 6.7. Effect of RIF on the *Rv1258c* promoter activity after 1, 2 and 24 hours of RIF (10 µg/ml) exposure for *rpoB* mutants harbouring the pMV306promo*lacZ* vector. The unexposed culture was exposed to DMSO (diluent of RIF). The β-galactosidase values represent the average for three independent experiments and error bars represent standard deviation.

6.4 DISCUSSION

The *M. tuberculosis* H37Rv *rpoB* mutations which occurred most frequently in this study were the H526Y (27.7%) mutation followed by the H526R (23.4%) and H526D (17%) mutations. The S531L mutation occurred at a frequency of only 9.6%. It has previously been

observed that the H526Y mutation occurred more frequently than the S531L mutant in another smaller in-house library of another strain of *M. tuberculosis* H37Rv *rpoB* mutants. In fact, no S531L mutant could be found among the *rpoB* mutants in the previous library while multiple H526Y mutants were present. Huitric *et al.* (2006) investigated the frequencies of spontaneous *rpoB* mutations in the RRDR *in vitro* in *M. tuberculosis* with a Beijing and non-Beijing genotype and observed the S531L mutation most frequently, followed by the H526Y mutation. The S531L, D516V and H526D mutations were the most frequently occurring *rpoB* mutations in Latvian isolates of MDR *M. tuberculosis* (Tracevska *et al.*, 2002). In Syrian *M. tuberculosis* clinical isolates the S531L mutation also occurred most frequently (Madania *et al.*, 2012). Interestingly, from the above studies the S531L mutant appears to occur more frequently in the clinical setting than the H526Y mutant. This is hypothesized to be due to the lower fitness cost associated with the S531L mutation which had no fitness cost in some clinical isolates (Gagneux, 2009). The S531L mutant was also associated with the lowest fitness cost in laboratory derived mutants of two different pan-susceptible *M. tuberculosis* strains (Gagneux, 2009). The fitness cost for the H526Y mutant was also low in the laboratory derived mutant, but not as low as the S531L mutant (Gagneux, 2009).

It is not clear why the mutation distribution in this study differed from previous observations. The fitness cost associated with the *rpoB* mutation was determined to be dependent not only on the type of *rpoB* mutation, but also on the strains genetic background (Gagneux, 2009). This could explain why the distribution of *rpoB* mutants was different in this study. There may also have been a bias in the colony selection. No novel mutations were discovered as all the mutations observed had been recorded before (Telenti *et al.*, 1993). Interestingly, for the integrating vector transformed strains, the H526Y mutant had a tendency to grow slower than the wild-type indicating a slight fitness cost under culturing conditions. The S531L mutant tended to grow faster than the wild-type although there were no real differences in the growth of the wild-type and S531L and H526Y *rpoB* mutants (Fig 6.5). This seems contradicting to the frequency of the mutants since fitness cost in terms of growth of the S531L mutant seemed to be lower than that of the H526Y mutant. This could be because of different growth conditions on 7H10 OADC media containing RIF (2 µg/ml) during selection of mutants and in 7H9 ADC in the absence of RIF when culturing the mutants.

The RIF MIC for the S531L and H526Y mutants were between 150 and 200 µg/ml and equal to or greater than 200 µg/ml respectively. Studies have indicated that the S531L *rpoB*

mutations are associated with high-level resistance in *M. tuberculosis* clinical isolates with a Beijing and non-Beijing genotype (Huitric *et al.*, 2006), in *M. tuberculosis* clinical isolates from Western Japan (Ohno *et al.*, 1996) and in clinical isolates from Poland (Zaczek *et al.*, 2009). The H526Y mutation was associated with high level resistance in the *M. tuberculosis* clinical isolates from Western Japan, as these mutations were not frequently observed in the other two studies (Ohno *et al.*, 1996). Even though the H526Y mutant occurred much less frequently in the clinical setting, possibly due to a slight fitness cost in the human host, it was still associated with high-level RIF resistance. Bahrmand *et al.* (2009) also showed that multiple *rpoB* mutations correlated specifically with codons 531 and 526 and are associated with high level RIF resistance in secondary infection cases.

It was determined that the integrating vector was stably maintained in the absence of the selectable antibiotic, hygromycin for the duration of the culturing that would be done for subsequent β-galactosidase assays. This eliminates hygromycin as a possible factor influencing *Rv1258c* promoter activity. *Rv1258c* expression differs significantly between different *rpoB* mutants. The *Rv1258c* promoter activity for the S531L mutant was approximately half of that of the wild-type while the promoter activity for the H526Y mutant was approximately double that of the wild-type. Mutations in the *rpoB* gene has been shown to alter transcriptional termination (Yanofsky & Horn, 1981) and DNA supercoiling (Drlica *et al.*, 1988) and cause defects in transcription the sigma subunit of RNA polymerase in *E. coli* (Zhou & Jin, 1997) and in the process alter gene expression.. Besides from the RIF resistance phenotype associated with the *rpoB* mutations in *B. subtilis*, each *rpoB* mutation differentially affected gene expression and had their own unique effect on phenotype (Maughan *et al*., 2004; Perkins & Nicholson, 2008; Moeller *et al.*, 2012). The *rpoB* mutations also affected antibiotic production in *S. lividans* and *S. coelicolor* (Hu & Ochi, 2001; Hu *et al*., 2002). Some of these affects might also be present in other bacteria including *M. tuberculosis* together with other unknown effects of *rpoB* mutations on gene expression (as this has not been studied extensively in *M. tuberculosis*). The mutation in *rpoB* could change the structure of the RNA polymerase that in turn could changes the binding of the RNA polymerase to the promoter regions. It is possible that binding of RNA polymerase to different transcription factors involved in the expression of *Rv1258c* are influenced to a different extent by the S531L and H526Y mutations causing differential expression of the *Rv1258c* promoter. However, this needs to be investigated.

The MIC for the H526Y mutant was slightly higher than that of the S531L mutant while the expression levels of *Rv1258c* was much higher (approximately four times) in the H526Y mutant than that observed for the S531L mutant. It is therefore possible that *Rv1258c* could be a contributor to the higher MIC observed in the H526Y mutant. In, *C. jejuni* ciprofloxacin, nalidoxic acid and enrofloxacin resistance was associated with *gyrA* mutations as well as the expression of the *cmeABC* gene encoding an efflux pump. Upon inactivation of the CmeABC efflux pump there were differences in the fold reduction in ciprofloxacin, nalidoxic acid and enrofloxacin resistance between different *gyrA* mutants. Interestingly, there were also differences in the fold reduction in ciprofloxacin MIC in isolates with the same *gyrA* mutations. This indicates that not only the mutations themselves play a role in the level of efflux pump expression and contribution to the MIC, but other mutations in the genome might also influences the expression levels of efflux pumps (Luo *et al.*, 2003).

The contribution of efflux to fluoroquinolone resistance for different ofloxacin resistant clinical isolates of *M. tuberculosis* with different *gyrA* mutations conferring ofloxacin resistance was investigated by Singh *et al.* (2011). The fold decrease in MIC for ofloxacin was investigated in the presence of efflux pump inhibitors. The contribution of efflux to the level of resistance differed between different *gyrA* mutants, suggesting that there may be differential efflux pump expression in different *gyrA* mutants. However, there were also differences observed in the MIC in the presence of efflux pump inhibitors between clinical isolates with the same *gyrA* mutations, indicating that other mechanisms are also involved (Singh *et al.*, 2011). These studies support the hypothesis that efflux pumps contribute to resistance in the presence of mutations in target genes. It is therefore plausible to suggest that the differential expression of *Rv1258c* observed in this study for the S531L and H526Y mutants may have an influence on the MIC for RIF in these strains. It is possible that efflux might contribute to a different extent to RIF resistance associated with the S531L and H526Y mutants.

Although it was assumed that the only mutations present in the *in vitro* generated mutants were the *rpoB* mutations, there is always a possibility that other mutations could have occurred concurrently elsewhere in the genome during the selection, culturing and transformation of the *rpoB* mutants. It has been observed in our group that random single nucleotide polymorphisms (SNPs) can occur spontaneously upon a single sub-culture, which

makes this assumption flawed in some ways. It was for this reason that sub-culturing was minimalized during the preparation of the tranformants. Whole genome sequencing would need to be done in order to prove that no other mutations had occurred which could possibly be implicated in the different *Rv1258c* promoter activity levels. However, had spontaneous mutants occurred which influenced the results of this study; it is highly unlikely that the same spontaneous mutations occurred in both the *M. smegmatis* and *M. tuberculosis* mutants and transformants. The results obtained for both *M. smegmatis* and *M. tuberculosis* were highly comparable and it is for this reason highly unlikely that some spontaneous SNP other than the SNP in *rpoB*, occurring elsewhere in the genome, is the cause of the differences in *Rv1258c* expression levels in the S531L and H526Y mutants.

Exposure of the S531L and H526Y mutants to critical concentration of RIF did not affect *Rv1258c* expression in either of the *rpoB* mutants. Increased expression of *Rv1258c* was previously observed in an *in vitro* generated *M. tuberculosis* H37Rv RIF mono-resistant mutant (Sharma *et al.,* 2010). It is important to note that there were significant differences between the aforementioned study and this study.

In the Sharma *et al.* (2010) study, a *M. tuberculosis* H37Rv spontaneous RIF resistant mutant was selected on 2 μg/ml RIF and the MIC for the RIF resistant strain was determined to be 128 μg/ml. The MIC for the sensitive parental strain was determined to be 0.25 μg/ml. The sensitive strain and the RIF resistant strain were cultured in the presence of RIF at a concentration of one fourth of their respective RIF MIC until mid-log phase, whereas in this study the mutants were cultured to early log phase and RIF added at that stage. Therefore the concentration of RIF to which the resistant mutant was exposed to was higher and the duration of exposure to RIF longer as compared to the current study.

It is possible that in this study, the time allowed for exposure to RIF was not sufficient to induce *Rv1258c* expression. However, in a study done to determine the rate of accumulation of RIF (C^{14} at 2 μg/ml) by *M. tuberculosis*, a steady state concentration of 154 μg/ml RIF was reached in the cells after only one to 2 min of RIF exposure. This steady state concentration was maintained over the next 20 min (Piddock *et al.*, 2000). This suggests that the RIF would have been present in the cells within minutes of exposure. It seems unlikely that the bacteria would only induce efflux pump expression after several days of exposure to a foreign substance. In the same instance, only increasing efflux pump expression after hours of

exposure might be too late to counter any negative effects that RIF might have even on RIF resistant strains. It has been suggested that the efflux pumps might serve as one of the first mechanisms of resistance and provide the bacteria with sufficient time to acquire drug resistance mutations, which presumably is an immediate response. This however may not be the case in strains already containing a RIF resistance mutation. It is thus still unclear why *Rv1258c* promoter expression was not upregulated in this study after hours of RIF exposure or why it was upregulated in the Sharma *et al.* (2010) study after days of RIF exposure.

It was previously shown that there was an increase in the concentration of RIF accumulated within the cells upon exposure to an increased concentration of RIF (C^{14}) in *M. tuberculosis* (Piddock *et al.*, 2000). Thus, it is possible that the accumulated RIF at 2 µg/ml RIF was not sufficient to induce *Rv1258c* expression, possibly because of the high RIF MIC for the mutants used in this study. In *M. tuberculosis* MDR clinical isolates efflux pump inhibitors reserpine and verapamil restored RIF susceptibility. However, in RIF pan-susceptible *M. tuberculosis* clinical isolates the RIF resistance was not changed by the addition of efflux pump inhibitors reserpine and verapamil. This suggests that efflux pumps which are active in the resistant strains (and are targeted by reserpine and verapamil) are not active in the pan-susceptible strain (Louw *et al.* 2011). This indicates a possible role of the level of RIF a strain is able to tolerate (RIF pan-susceptible versus RIF resistant) in the induction of pumps, since the mechanism may be related to the toxic effects of RIF on the cell. It would be interesting to observe the effects of adding a concentration of RIF of approximately one fourth of the MIC for the S531L and H526Y mutants and observing whether *Rv1258c* is induced. We chose to use 2 µg/ml in this study since it is a clinically relevant concentration while one fourth of the MIC of the mutants is not since such high concentrations of RIF would never be reached in a clinical setting. However, it was suggested that RIF is rapidly removed by efflux from the cell upon RIF exposure and that a high RIF concentration (higher than critical concentration of 2 µg/ml) is thus needed for binding of the mutant RpoB to RIF to occur and thus for RIF to have its effects (Louw *et al.*, 2011).

Several studies using clinical isolates of *M. tuberculosis* showed induction of *Rv1258c* following exposure to RIF. Gupta *et al.* (2006) showed that *Rv1258c* expression was increased to the same extent upon exposure to RIF (1 µg/ml), isoniazid, streptomycin, ofloxacin and ethambutol in clinical isolates. These isolates were cultured to early log phase and the drugs added. After incubation of 16-18 hours in the presence of drug, *Rv1258c*

expression was assessed (Gupta *et al.*, 2006). Unfortunately, the RIF MICs for the strains used were not provided in the article.

Rv1258c expression was also increased upon exposure to RIF and ofloxacin in the *M. tuberculosis* clinical isolate ICC154. ICC154 is resistant to RIF (MIC equal to 40 µg/ml), ofloxacin (MIC equal to 4 µg/ml), isoniazid (MIC equal to 2 µg/ml) and minomycin (MIC equal to 2µg/ml). The isolate was cultured in the presence of sub-lethal concentrations of one fourth the MIC for RIF and ofloxacin respectively. RNA was extracted and *Rv1258c* expression examined. There was a 10 fold increase in *Rv1258c* expression in the presence of RIF and six fold increase in the presence of ofloxacin (Siddiqi *et al.*, 2004). *Rv1258c* expression was also increased upon RIF and INH exposure in the *M. tuberculosis* multidrug-resistant strain 1499. The 1499 strain harboured mutations in *rpoB* (S531L) and *katG* genes and had a MIC of 6.4 µg/ml for RIF and 102.4 µg/ml for INH (Jiang *et al.*, 2008). The strain was also cultured in the presence of one fourth of the RIF and INH MIC respectively. *Rv1258c* expression was increased three fold in the presence of RIF and six fold in the presence of INH (Jiang *et al.*, 2008).

Another possible explanation for the lack of upregulation following RIF exposure is that it is related to differences in the MIC of the isolates. However, upon knocking out the *Rv1258c* gene in *M. tuberculosis* H37Rv RIF resistant strain, there was no decrease in MIC observed for the knockout when compared to that of the wild-type strain (Villellas, 2012). This indicates that *Rv1258c* was not contributing to the MIC for RIF in the particular H37Rv RIF resistant strain used in that study. In the aforementioned Siddiqi *et al.* (2004) and Jiang *et al.* (2008) studies, where up-regulation was observed, the MICs were relatively low at 40 µg/ml and 6.4 µg/ml respectively. Furthermore, *Rv1258c* was shown to only be upregulated in Beijing strains with low level RIF resistance and not those with high resistance level RIF resistance (Louw *et al.*, 2011). It is therefore possible that the concentration of RIF which a strain can tolerate affects the ability of RIF to induce *Rv1258c* expression. This would suggest induction of *Rv1258c* is due to a general stress response to the presence of the "foreign" RIF molecule. Strains with a higher RIF MIC would therefore require a higher RIF concentration to induce this stress response. This hypothesis is supported by the observation by Gupta *et al.* (2006) that several drugs were able to induce this *Rv1258c*, presumably by a common mechanism. Alternatively, strains with a high RIF MIC may have maximum

constitutive expression of *Rv1258c*, which cannot be further induced in response to RIF exposure.

Interestingly, the Tap efflux pump (an *Rv1258c* homologue) in *M. bovis* was shown to fulfil physiological functions during stationary growth (Ramón-García *et al.*, 2012). Kyselková *et al.* (2012) compared the presence of tetracycline resistance and tetracycline resistance genes including the gene encoding the Tap efflux pump, in soil mycobacteria to that of the clinically resistant mycobacteria. The Tap efflux pump was shown to be present in both soil mycobacteria and clinically relevant mycobacteria and was present in both drug resistant and non-resistant mycobacterial species indicating a possible role of Tap in intrinsic tetracycline resistance (Kyselková *et al.*, 2012). These studies suggest a physiological role of *Rv1258c* in *M. tuberculosis*. Overexpression of MFS efflux pumps for which the energy source is provided by a proton gradient, could cause the accidental loss of important metabolites and loss of membrane potential, which could ultimately lead to cell death (Nikaido, 1998; Grkovic *et al.*, 2002). These are reasons why the expression of *Rv1258c* would need to be tightly regulated and could explain why further upregulation of *Rv1258c* following exposure to a low RIF concentration in a strain which has a high MIC does not occur.

It is also possible that the strains in which upregulation is observed share a common, unknown mechanism which is responsible for regulating *Rv1258c* expression in response to RIF, not present in the strains used in this study or in other strains in which upregulation was not observed. It would be of interest to whole genome sequence both H37Rv strains, the one used in this study and the one used in the Sharma *et al.* (2010) study, which could have different SNPs elsewhere in the genome to see which SNPs are present and could possibly help explain why these two H37Rv strains responded differently to exposure to RIF. The upregulation in *Rv1258c* expression in some genetically clustered strains, but not in others could be explained by other SNPs present in the strains.

When examining the genes upregulated upon exposure to critical concentration (2 µg/ml) of RIF, *Rv1258c* expression was upregulated in strains with a low copy clade genotype which have both low and high levels of RIF resistance respectively and in strains with a Beijing genotype and low level RIF resistance (Louw *et al.*, 2011). However, *Rv1258c* expression was not upregulated upon RIF exposure in the strains with a Beijing genotype and high level RIF resistance as mentioned before (Louw *et al.*, 2011). If other SNPs are affecting *Rv1258c*

expression, you would expect to see difference in the expression in the absence of RIF as well as differences in the response of the strains to the exposure to RIF in terms of *Rv1258c* expression. The other SNPs present in the strains could possibly explain why *Rv1258c* was not upregulated upon exposure to RIF in this study while it was upregulated in another *M. tuberculosis* H37Rv strain upon exposure to RIF in the Sharma *et al.* (2010) study and in the multidrug resistant clinical isolates.

The presence of *rpoB* mutations affects *Rv1258c* promoter expression in *M. tuberculosis* with a lower expression observed in the S531L mutant and higher expression in the H526Y mutant. RIF did not induce *Rv1258c* promoter expression in the strains used in this study.

CHAPTER 7: DO β-GALACTOSIDASE ASSAYS REPRESENT *RV1258C* TRANSCRIPT LEVELS AND WHAT IS THE EFFECT OF *RPOB* MUTATIONS ON *WHIB7* TRANSCRIPT LEVELS?

7.1 INTRODUCTION

Promoter activity assays are a convenient way for determining conditions which influence gene expression. We wanted to determine whether *Rv1258c* promoter activity observed using the promoter reporter assays correlated to the transcript levels of the native intact *Rv1258c* gene. qRT-PCR to determine the native *Rv1258c* gene transcript levels in the wild-type and *rpoB* mutants of *M. tuberculosis* was done.

Comparison of gene expression between samples by qRT-PCR requires a reference gene to which expression is normalized, to correct for variation in the amount of RNA in each sample. The *sigma factor A* (*sigA*) gene was chosen as a reference gene because the *M. tuberculosis* SigA is presumably the principal sigma factor, as the *sigA* homologue of *M. smegmatis* has been determined to be indispensable (Gomez *et al.*, 1998). It has also been shown that the *sigA* gene is constitutively expressed under most of the conditions tested by Manganelli *et al.* (1999). The *sigA* gene has also been used before in the literature as a housekeeping gene in studies which examined *Rv1258c* expression with qRT-PCR (Sharma *et al.*, 2010) and the gene encoding the Tap efflux pump (a *Rv1258c* homologue) in *M. bovis* BCG (Ramón-García *et al.*, 2012). Other genes such as *Rv1437* (encoding phosphoglycerate kinase) and 16S rRNA have also been used as housekeeping genes when studying *Rv1258c* expression (Siddiqi *et al.*, 2004; Jiang *et al.*, 2008). Also, the *sigA* gene has been used as housekeeping gene before to study differences in gene expression between *M. tuberculosis* wild-type and a *rpoB* mutant strain (Bisson *et al.*, 2012). The *sigA* gene has also been used before in the literature as housekeeping gene when investigating *whiB7* expression (Morris *et al.*, 2005; Geiman *et al.*, 2006).

As a second aim of the project we wanted to determine the effects of the *rpoB* mutations on the expression of *whiB7*. As discussed in the literature review, *whiB7* encodes a global transcriptional regulator (WhiB7). The expression of *Rv1258c* was found to be dependent on the initial induction of *whiB7*, following exposure to antibiotics (Morris *et al.*, 2005). The

whiB7 gene expression was induced in the presence of drugs such as kanamycin, cycloserine and streptomycin (Geiman *et al.*, 2006). WhiB7 thus plays a role in drug resistance.

7.2 METHODOLOGY

7.2.1 RNA extraction and RNA concentration determination

During preparation of WCLs for the β-galactosidase assays, RNA was simultaneously extracted from the *M. tuberculosis* H37Rv wild-type and *rpoB* mutants harbouring the pMV306promo*lacZ* vector cultures at the 24 hour time point using the RNA ProBlue kit. Two RNA extractions were done in parallel from each culture.

The Experion RNA StdSens Analysis kit (Bio-Rad) was used to determine the RNA concentration and purity according to the manufacturer's instruction. Briefly, the kit reagents (RNA stain, RNA loading buffer and RNA gel) were equilibrated to room temperature and the RNA gel was filtered. The Experion electrode was cleaned with electrode cleaner followed by two rinsing steps with water. RNA stain (1 µl) was added to filtered gel (65 µl) and will subsequently be called the gel stain solution. The chip was primed with gel stain solution. The gel stain solution, filtered gel and loading buffer were loaded into the wells of the chip. The RNA samples (2 µl) and the RNA ladder (2 µl) were denatured for 2 min at 70°C and placed on ice immediately afterwards. RNA samples and RNA ladder were loaded into the appropriate wells. The chip was vortexed for 10 seconds and placed in the Experion electrophoresis station (Bio-Rad). Experion software (Bio-Rad) was used for analysis.

7.2.2 cDNA synthesis

Reverse transcription was done using three gene specific primers that reverse transcribe *sigA*, *Rv1258c* and *whiB7* using the MTB-sigA-RT, Rv1258c-RT and whiB7-RT primers respectively (Table 3.4). cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Sciences) according to the manufacturer's instructions. Firstly, RNA (1,500 µg) was added to a final concentration of 2 µM of each of the MTB-sigA-RT, Rv1258c-RT and whiB7-RT primers and made up to a final volume of 13 µl with water. The RNA primer mix was denatured for 10 min at 65°C in a heated thermal block cycler and placed on ice. The 1× transcriptor reverse transcriptase buffer, protector RNase

inhibitor (20 U), dNTP (final concentration of 1 mM each) and transcriptor reverse transcriptase (10 U) were added (making the total volume of each cDNA synthesis reaction 20 µl). After mixing, the samples were incubated at 55°C for 30 min in a thermal block cycler. Inactivation of the enzyme was done by incubating at 85°C for 5 min. The cDNA was stored at -20°C until later use for qRT-PCR.

7.2.3 DNA dilutions for standard curve

Genomic DNA from the *M. tuberculosis* H37Rv Sasetti strain was provided by M. Williams. The concentration of the DNA was determined using the Nanodrop and gel quantification. In order to prepare a standard curve, a dilution was made with a final concentration of 10^7 *M. tuberculosis* genome copies per 2 µl. The concentration of DNA that represents 10^7 *M. tuberculosis* genome copies per 2 µl was determined as illustrated in Addendum C. The dilution of 10^7 genomes per 2 µl was aliquoted and stored at -20°C. A dilution range of this (10^6 to 10^3) was prepared fresh for every qRT-PCR experiment and each plate.

7.2.4 qRT-PCR

The FastStart Universal SYBR Green Master (Rox) kit (Roche Applied Sciences) was used for qRT-PCR according to the manufacturer's instructions. The standard curve method for relative quantification of *Rv1258c* and *whiB7* expression relative to the *sigA* gene was used. Three mixes, each containing the set of primers for one of the genes were prepared. The final mixes contained: 1× FastStart Universal SYBR Green Master (Rox) mix, 300 nM forward and reverse primers (MTB-sigA-F1 and MTB-sigA-R1 or Rv1258c-F1 and Rv1258c-R1 or whiB7-F1 and whiB7-R1) in a total reaction volume of 50 µl.

The reaction mixes were prepared and aliquoted into a MicroAmp® Optical 96-Well Reaction Plate (100 µl) (Applied Biosystems) and the wells were closed with strip caps (Applied Biosystems). The plate was covered with paper towel and kept on ice until used. The genomic DNA (10^7 genomes per 2 µl) stocks were defrosted on ice and a dilution series prepared (10^6 to 10^3). This dilution range was previously determined to fit onto the linear range. The cDNA samples were defrosted on ice. Dilutions (tenfold and 100 fold) of the cDNA were also prepared in order to ensure that the samples' cycle threshold (C_t) values could be read from the standard curve. The DNA dilution series and undiluted and diluted

cDNA (2 µl) were added to their respective wells. Each sample was read in technical duplicate. Three independent experiments representing three biological replicates were done. Melting curve analysis was also done.

The qRT-PCR was done on an Applied Biosystems StepOnePlus Real-Time PCR machine (Applied Biosystems) and using the StepOne software (Applied Biosystems). The run protocol was as follows: Activation of FastStart Taq DNA polymerase for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing and elongation at 61 °C for 60 s. The Ct values of the DNA dilution series was plotted against the log genome copy number for the *sigA*, *Rv1258c* and *whiB7* genes. A trend line was set to the values which fall within the linear range for each gene. The gradient of the trend line was used to determine the log copy number using the average Ct values of each of the technical duplicate cDNA samples. The copy number was determined from the log copy number. The copy numbers determined for the *Rv1258c* and *whiB7* genes were divided by the copy numbers of *sigA* in the same sample. The results for three independent experiments or biological triplicates were averaged and the standard deviation determined. The expression levels of *Rv1258c* and *whiB7* normalised to *sigA* expression were compared between the wild-type, S531L and H526Y *rpoB* mutants.

7.3 RESULTS

The levels of the native *Rv1258c* gene expression at transcript level were compared between the wild-type and *rpoB* mutants using qRT-PCR in order to determine whether the results obtained during β-galactosidase assays were representative of the abundance of native gene transcripts. The effects of *rpoB* mutations on *whiB7* expression were also determined using qRT-PCR.

7.3.1 RNA concentration determination using Experion RNA StdSens Analysis kit

Since the RNA concentration determined using the Nanodrop might not be accurate, the RNA concentration and purity was determined using the Experion RNA StdSens analysis kit (Bio-Rad) and software. A representative gel is presented in Fig 7.1.

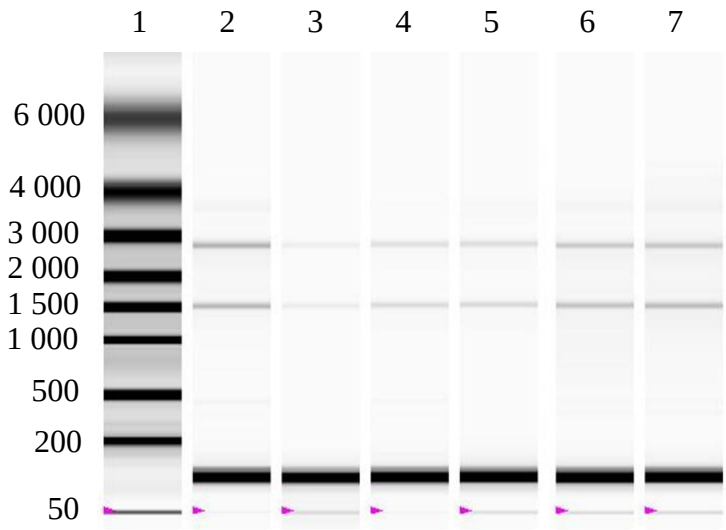


Figure 7.1. Representative Experion RNA gel. Lane (1) RNA ladder, lanes (2-7) representative RNA samples.

7.3.2 qRT-PCR

The expression of *sigA* in the wild-type and *rpoB* mutants was investigated first in order to determine whether the expression of *sigA* between the strains were comparable and could be used as housekeeping gene. There was no difference in the *sigA* transcript copy numbers between the wild-type and *rpoB* mutants (Table 7.1). This means that *sigA* could be used as housekeeping gene.

Table 7.1. Number of transcript copies of *sigA* in the wild-type and *rpoB* mutants. The values represent the average of three independent experiments ± standard deviation.

Strain	**Transcript copies of *sigA***
Wild-type	352,175±217,374
rpoB S531L mutant	323,936±130,614
rpoB H526Y mutant	381,921±117,149

The innate *Rv1258c* gene expression at transcript level was investigated using qRT-PCR. The expression levels of *whiB7* were also investigated using qRT-PCR in order to determine the effect of *rpoB* mutations on *whiB7* expression. The qRT-PCR results are summarised in Table 7.2.

The differences in *Rv1258c* expression was not significant but was only marginal with a similar trend to those seen with the promoter reporter studies. The S531L mutant showed marginally lower expression and H526Y mutant marginally higher expression than the wild-type. Expression of *whiB7* in the *rpoB* mutants did not differ significantly from those observed for the wild-type. However, *whiB7* expression did show a similar trend to the trend observed in *Rv1258c* transcript levels and promoter activity, as the expression tended to be lower in the S531L mutant and higher in the H526Y mutant compared to the wild-type. The expression of *whiB7* in the S531L mutant was significantly lower than that of the H526Y mutant (p=0.0129).

Table 7.2. The relative expression levels of *Rv1258c* and *whiB7* relative to *sigA* in the pMV306promo*lacZ* transformed *M. tuberculosis* H37Rv wild-type and *rpoB* mutant strains. The values represent the average of three independent experiments ± standard deviation. Significant differences in expression are indicated by * representing p<0.05.

	Expression levels of transcript relative to *sigA*	
Strain	***Rv1258c***	***whiB7***
Wild-type	0.59±0.24	1.00±0.19
rpoB S531L mutant	0.56±0.27	0.71±0.04 *
rpoB H526Y mutant	0.67±0.26	1.10±0.15 *

7.4 DISCUSSION

Although differences were not significant, *Rv1258c* transcript expression followed the same trend as those obtained for the β-galactosidase assay as there was a marginally lower *Rv1258c* transcript level in the S531L mutant than in the wild-type while the *Rv1258c* transcript level was marginally higher in the H526Y mutant. The differences in *Rv1258c* expression levels appeared to be much bigger (significant differences observed) when measured as promoter activity in β-galactosidase assays than it appeared at transcript level during qRT-PCR (no significant differences observed). This could be because any small differences in expression are amplified in the β-galactosidase assays because of the nature of the assays. The use of β-galactosidase assays could thus be advantageous in that any small differences in expression levels of a gene could be picked up much more easily, however also cause problems since a

seemingly large difference observed during β-galactosidase assays might not actually represent a biologically significant difference in *Rv1258c* expression.

Furthermore, whether the difference observed in the *Rv1258c* promoter expression between the *rpoB* mutants and wild-type during β-galactosidase assays are biologically significant is unknown. In order to determine whether the differences in *Rv1258c* expression between the *rpoB* mutants are biologically significant, one would need to prove that *Rv1258c* activity is decreased in the S531L mutant while *Rv1258c* activity is increased in the H526Y mutant. Doing this would however be impossible, as the activity of *Rv1258c* alone cannot be measured in a system where other efflux pumps are also present.

It might be possible that the expression levels at promoter activity level for the *Rv1258c* promoter region fused to *lacZ* is not comparable to that of the native *Rv1258c* gene transcripts since the genes are in different contexts. It is also possible that mutating *rpoB* could change the structure of RpoB, which in turn could affect transcription of multiple genes. Differential expression of multiple genes could in turn affect the physiological state of the mutants, which in turn could indirectly alter the expression or regulation of *Rv1258c*.

When comparing the transcript levels of *whiB7* no significant difference between the wild-type and *rpoB* mutants were observed. A similar trend to that observed for *Rv1258c* transcript levels and promoter activity was however observed as expression in the S531L mutant was lower and in the H526Y mutant was higher than that of the wild-type. *Rv1258c* expression might be altered by the differential expression levels of *whiB7*. The exact mechanism of this is however unknown since transcript levels of *Rv1258c* were not affected.

CONCLUSIONS AND FUTURE STUDIES

During these past few years it has become clear that drug resistance in TB is more complicated than originally thought. It is generally believed that mutations in *rpoB* are associated with RIF resistance. However, there is now compelling evidence that efflux also plays an important role in determining the level of RIF resistance. The relationship between these mechanisms is not well understood. In this study it was hypothesised that *rpoB* mutations affect the expression of the *Rv1258c* efflux pump and the *whiB7* gene.

Promoter reporter assays are an established method for studying gene expression and we have taken advantage of this to investigate the effects of *rpoB* mutations on *Rv1258c* expression. The promoter region of *Rv1258c* was successfully cloned into an episomal vector and integrating vectors were also successfully constructed. The determined position of the transcriptional start site of *Rv1258c*, at six base pairs upstream of the annotated start codon, was questioned due to the apparent lack of a ribosomal binding site. As a future study, the vectors constructed in this study could be used to determine or confirm the position of the transcriptional start site determined in this study. This would involve mutagenesis of the *Rv1258c* promoter region which has been cloned into the reporter construct and from the promoter activity observed for the vector deducting whether the transcriptional start site is present or not.

Mutations in the *rpoB* gene cause altered *Rv1258c* promoter activity in *M. tuberculosis* H37Rv *in vitro* generated mutants. No significant difference in *Rv1258c* and *whiB7* transcript levels were however observed even though the same trend in expression as observed for the β-galactosidase assays was present. Whether the difference in *Rv1258c* expression between wild-type and *rpoB* mutants is biologically significant, remains to be determined.

It is possible that the expression levels at promoter activity level for the *Rv1258c* promoter region fused to *lacZ* is not comparable to that of the native *Rv1258c* gene transcripts since the genes are in different contexts. It would therefore be interesting to examine the transcript and protein levels of the *Rv1258c* promoter fused to *lacZ* and see whether there are differences at transcript or protein level between the wild-type and *rpoB* mutants. It would also be interesting to determine and characterize the regulator of *Rv1258c* in order to understand the regulation of *Rv1258c* to the full extent.

Exposure of the *M. smegmatis* and *M. tuberculosis rpoB* mutants to sub-inhibitory levels of RIF did not affect *Rv1258c* promoter activity. RIF does not affect the expression of *Rv1258c* in *M. smegmatis* possibly due to other mechanisms of resistance to RIF present in *M. smegmatis* or the RIF concentration not being high enough to induce *Rv1258c* expression in the *M. smegmatis rpoB* mutants. It is also possible that the concentration of RIF used (2 µg/ml) was not high enough to induce the expression of *Rv1258c* in *M. tuberculosis* since the MICs for the mutants were already so high. Alternatively, the expression of *Rv1258c* was already maximal and was thus not further induced.

It is also possible that the MIC of the strain influences the induction of *Rv1258c*. The effects of RIF MIC on *Rv1258c* expression should be investigated. This could be done by using *rpoB* mutants which have the same *rpoB* mutation and no other difference in SNPs, but different MICs and comparing *Rv1258c* expression levels. *Rv1258c* expression levels should also be investigated in other *rpoB* mutants generated in this study to determine how other *rpoB* mutations affect *Rv1258c* expression. This will help establish a relationship between the type of *rpoB* mutation and the level of *Rv1258c* expression.

The contribution of efflux, specifically *Rv1258c* expression, to the RIF MIC in the *M. tuberculosis* S531L and H526Y mutants could be assessed by adding efflux pump inhibitors to the MGIT cultures and determining the change in MIC. This would indicate whether efflux pumps contribute to a greater extent to the drug resistance (MIC) in the H526Y mutant than in the S531L mutant. The limitation of this approach is that there are many other MFS efflux pumps that might also be responsible for efflux of RIF, which will also be influenced by adding an efflux pump inhibitor.

It might also be possible that the time of exposure to RIF was not sufficient for induction of the *Rv1258c* promoter activity and that *Rv1258c* expression at different RIF exposure time points should have been investigated. It would be interesting to monitor *Rv1258c* expression at different exposure times ranging from min to hours to days of RIF exposure. This would help to show whether the time of exposure to RIF influences the expression of the *Rv1258c* efflux pump.

Since in previous studies *Rv1258c* was only upregulated upon exposure to RIF in some *in vitro* generated RIF mono-resistant *rpoB* mutants and MDR clinical isolates, but not in

others, the effects of other SNPs on *Rv1258c* expression also needs to be investigated. To do this *Rv1258c* expression levels between strains with different SNPs can be compared using the β-galactosidase assays as used in this study. The constructs generated in this study could provide a valuable tool for studying the expression of *Rv1258c* under various conditions and in various strains. However, one would need to be cautious when interpreting the results of β-galactosidase assays since a seemingly big difference in *Rv1258c* expression might not be biologically significant.

With drug resistance being such a major problem worldwide, new drug targets such as efflux pumps are needed. Efflux pumps are promising targets. When considering efflux pumps as targets for treatment, it needs to be taken into consideration that the expression of efflux pumps could be different because of differences in *rpoB* mutations present in drug resistant strains. With the increased occurrence of drug resistant *M. tuberculosis*, fighting back by using a combination of different mechanisms to acquire resistance, it is compulsory that we understand these mechanisms as well as their interconnectedness. This could provide us with the necessary knowledge to combat drug resistant *M. tuberculosis*.

SHORTCOMINGS OF THIS RESEARCH

The position of the transcriptional start site for *Rv1258c* could not be definitely determined in the 5' RACE experiments done. This leads to uncertainty as to whether the promoter region cloned into the vectors contained the *Rv1258c* transcriptional start site. However, during the experiment there was an increase in β-galactosidase activity in the promoter region containing vectors, and this increase was different for the two *rpoB* mutants in both *M. smegmatis* and *M. tuberculosis*, which indicates that a promoter was present in the promoter region amplified from *M. tuberculosis* H37Rv. It was however questioned whether the promoter region was in fact that of the *Rv1258c* gene. To this we counter argue based on the layout of the genes surrounding the *Rv1258c* gene, the promoter region of *Rv1258c* should be present somewhere upstream of this gene and this gene is the only gene in the vicinity on the reverse strand.

The *lacZ* gene and promoter fused to the *lacZ* gene were cloned into the integrating vector in a reverse orientation. These vectors were used in this study. Vectors in which the *lacZ* and promoter fused to *lacZ* gene were cloned co-directionally with the hygromycin cassette of the vector should also have been isolated and used in parallel to determine the influence of other vector elements on promoter activity.

Since variation in the protocol and media might be present between different laboratories when determining the MIC of strains and a specific RIF MIC was not determined for the *M. smegmatis* mc^2155 *rpoB* mutants (RIF MIC was determined to be somewhere between 125 and 250 µg/ml), the RIF MIC for these strains should have been determined again in our laboratory. This would enable a more specific comparison of the RIF MIC of the *M. smegmatis* mc^2155 *rpoB* mutants and could provide more information on a possible relationship between RIF MIC and *Rv1258c* expression.

When investigating the growth of the transformed *rpoB* mutant strains in *M. smegmatis* and *M. tuberculosis* the measurement of colony forming units would have provided a more sound measure of growth than measuring OD, as was done in this study. The OD is not necessarily an indication of whether the bacteria are alive and culturable while the amount of colony forming units is.

The *M. tuberculosis* H37Rv wild-type strain and mutants generated in this study were not whole-genome sequenced. This leaves the possibility of other SNPs that might be present in the genome affecting the experimental results.

Since the RIF MIC for the *M. smegmatis* and *M. tuberculosis rpoB* mutants were high, the effect of a few more concentrations of RIF (in addition to the 10 and 2 µg/ml RIF used respectively in this study) on *Rv1258c* expression should have been tested. The effect of different RIF concentrations on *Rv1258c* expression and whether different RIF concentrations affect *Rv1258c* expression differently could have been established by doing so.

REFERENCES

Adams, K.N., Takaki, K., Connolly, L.E., Wiedenhoft, H., Winglee, K., Humbert, O., Edelstein, P.H., Cosma, C.L., & Ramakrishnan, L. 2011. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145(1), 39–53.

Agarwal, N., Raghunand, T.R., & Bishai, W.R. 2006. Regulation of the expression of *whiB1* in *Mycobacterium tuberculosis*: Role of cAMP receptor protein. *Microbiol-UK*. 152, 2749–2756.

Ahmed, M., Borsch, C.M., Taylor, S.S., Vázquez-Laslop, N., & Neyfakh, A.A. 1994. A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J. Biol. Chem.* 269(45), 28506–28513.

Alam, M.S., Garg, S.K., & Agrawal, P. 2007. Molecular function of WhiB4/Rv3681c of *Mycobacterium tuberculosis* H37Rv: A [4Fe-4S] cluster co-ordinating protein disulphide reductase. *Mol. Microbiol.* 63(5), 1414–1431.

Alam, M.S., Garg, S.K., & Agrawal, P. 2009. Studies on structural and functional divergence among seven WhiB proteins of *Mycobacterium tuberculosis* H37Rv. *FEBS J.* 276, 76–93.

Alekshun, M.N., & Levy, S.B. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: The *mar* regulon. *Antimicrob. Agents Chemother.* 41(10), 2067-2075.

Alguel, Y., Meng, C., Terán, W., Krell, T., Ramos, J.L., Gallegos, M., & Zhang, X. 2007. Crystal structures of multidrug binding protein TtgR in complex with antibiotics and plant antimicrobials. *J. Mol. Biol.* 369, 829–840.

Amaral, L., Cerca, P., Spengler, G., Machado, L., Martins, A., Couto, I., Viveiros, M., Fanning, S., & Pagès, J.M. 2011. Ethidium bromide efflux by *Salmonella*: Modulation by metabolic energy, pH, ions and phenothiazines. *Int. J. Antimicrob. Ag.* 38(2), 140–145.

Arjomandzadegan, M., Sadrnia, M., Surkova, L.K., & Titov, L.P. 2011. Study of promoter and structural gene sequence of *whiB7* in MDR and XDR forms of *Mycobacterium tuberculosis. West Indian Med. J.* 60(3), 251–256.

Bachas, S., Eginton, C., Gunio, D., & Wade, H. 2011. Structural contributions to multidrug recognition in the multidrug resistance (MDR) gene regulator, BmrR. *Proc. Natl. Acad. Sci. U.S.A.* 108(27), 11046–11051.

Bahrmand, A.R., Titov, L.P., Tasbiti, A.H., Yari, S., & Graviss, E.A. 2009. High-level rifampin resistance correlates with multiple mutations in the *rpoB* gene of pulmonary tuberculosis isolates from the Afghanistan border of Iran. *J. Clin. Microbiol.* 47(9), 2744–2750.

Balganesh, M., Dinesh, N., Sharma, S., Kuruppath, S., Nair, A.V., & Sharma, U. 2012. Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates. *Antimicrob. Agents Chemother*. 56(5), 2643–2651.

Balganesh, M., Kuruppath, S., Marcel, N., Sharma, S., Nair, A., & Sharma, U. 2010. Rv1218c, an ABC transporter of *Mycobacterium tuberculosis* with implications in drug discovery. *Antimicrob. Agents Chemother*. 54(12), 5167–5172.

Baranova, N.N., Danchin, A., & Neyfakh, A.A. 1999. Mta, a global MerR-type regulator of the *Bacillus subtilis* multidrug-efflux transporters. *Mol. Microbiol.* 31(5), 1549–1559.

Barbosa, T.M., & Levy, S.B. 2000. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. *J. Bacteriol.* 182(12), 3467–3474.

Bardouniotis, E., Ceri, H., & Olson, M.E. 2003. Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. *Curr. Microbiol.* 46, 28–32.

Bellinzoni, M., Buroni, S., Schaeffer, F., Riccardi, G., De Rossi, E., & Alzari, P.M. 2009. Structural plasticity and distinct drug-binding modes of LfrR, a mycobacterial efflux pump regulator. *J. Bacteriol.* 191(24), 7531–7537.

Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A., & Duncan, K. 2002. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* 43(3), 717–731.

Bisson, G.P., Mehaffy, C., Broeckling, C. 2012. Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampicin-resistant, *rpoB*-mutant *Mycobacterium tuberculosis*. *J. Bacteriol.* 194(23), 6441–6452.

Bolla, J.R., Do, S.V., Long, F., Dai, L., Su, C., Lei, H., Chen, X., Gerkey, J.E., Murphy, D.C., Rajashankar, K.R., Zhang, Q., & Yu, E.W. 2012. Structural and functional analysis of the transcriptional regulator Rv3066 of *Mycobacterium tuberculosis*. *Nucleic Acids Res.* 40(18), 9340–9355.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

Brooun, A., Liu, S., & Lewis, K. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 44(3), 640–646.

Burian, J., Ramón-García, S., Sweet, G., Gómez-Velasco, A., Av-Gay, Y., & Thompson, C.J. 2012. The mycobacterial transcriptional regulator *whiB7* links redox homeostasis and intrinsic antibiotic resistance. *J. Biol. Chem.* 287, 299–310.

Buroni, S., Manina, G., Guglierame, P., Pasca, M.R., Riccardi, G., & De Rossi, E. 2006. LfrR is a repressor that regulates expression of the efflux pump LfrA in *Mycobaterium smegmatis. Antimicrob. Agents Chemother.* 50(12), 4044–4052.

Campbell, E.A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., & Darst, S.A. 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell.* 104, 901–912.

Carroll, P., & James, J. 2008. Assaying promoter activity using LacZ and GFP as reporters. In Parish, T., & Brown, A.C. (eds.). *Mycobacteria protocols.* 2nd edition. pp. 265–277. Totowa, NJ: Humana Press.

Casonato, S., Sánchez, A.C., Haruki, H., González, M.R., Provvedi, R., Dainese, E., Jaouen, T., Gola, S., Bini, E., Vicente, M., Johnsson, K., Ghisotti, D., Palù, G., Hernández-Pando, R., & Manganelli, R. 2012. WhiB5, a transcriptional regulator that contributes to *Mycobacterium tuberculosis* virulence and reactivation. *Infect. Immun.* 80(9), 3132–3144.

Cerda-Maira, F.A., Ringelberg, C.S., & Taylor, R.K. 2008. The bile response repressor BreR regulates expression of the *Vibrio cholera breAB* efflux system operon. *J. Bacteriol.* 190(22), 7441–7452.

Chan, Y.Y., Bian, H.S., Tan, T.M.C., Mattmann, M.E., Geske, G.D., Igarashi, J., Hatano, T., Suga, H., Blackwell, H.E., & Chua, K.L. 2007. Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. *J Bacteriol*. 189(11), 4320–4324.

Chan, Y.Y., & Chua, K.L. 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: Expression and impact on quorum sensing and virulence. *J. Bacteriol.* 187(14), 4707–4719.

Chan, Y.Y., Tan, M.C., Ong, Y.M., & Chua, K.L. 2004. BpeAB-OprB, a multidrug efflux pump in *Burkholderia pseudomallei*. *Antimicrob. Agents Chemother.* 48(4), 1128–1135.

Chawla, M., Parikh, P., Saxena, A., Munshi, M., Mehta, M., Mai, D., Srivastava, A.K., Narasimhulu, K.V., Redding, K.E., Vashi, N., Kumar, D., Steyn, A.J., & Singh, A. 2012. *Mycobacterium tuberculosis* WhiB4 regulates oxidative stress response to modulate survival and dissemination *in vivo*. *Mol. Microbiol.* In press. doi: 10.1111/j.1365-2958.2012.08165.x.

Cohen, S.P., Hächler, H., & Levy, S.B. 1993. Genetic and functional analysis of the multiple antibiotic resistance *(mar)* locus in *Escherichia coli*. *J. Bacteriol.* 175(5), 1484–1492.

Colangeli, R., Helb, D., Vilchèze, C., Hazbón, M.H., Lee, C., Safi, H., Sayers, B., Sardone, I., Jones, M.B., Fleischmann, R.D., Peterson, S.N., Jacobs Jr., W.R., & Alland, D. 2007.

Transcriptional regulation of multi-drug tolerance and antibiotic-induced responses by the histone-like protein Lsr2 in *M. tuberculosis. PLoS Pathog.* 3(6), 0780–0793.

De Kievit, T.R., Parkins, M.D., Gillis, R.J., Srikumar, R., Ceri, H., Poole, K., Iglewski, B.H., & Storey, D.G. 2001. Multidrug efflux pumps: Expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 45(6), 1761–1770.

De Rossi, E., Arrigo, P., Bellinzoni, M., Silva, P.E.A., Martín, C., Aínsa, J.A., Guglierame, P., & Riccardi, G. 2002. The multidrug transporters belonging to major facilitator superfamily (MFS) in *Mycobacterium tuberculosis*. *Mol. Med.* 8(11), 714–724.

De Rossi, E., Branzoni, M., Cantoni, R., Milano, A., Riccardi, G., & Ciferri, O. 1998. *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. *J. Bacteriol.* 180(22), 6068–6071.

Deng, X., Sun, F., Ji, Q., Liang, H., Missiakas, D., Lan, L., & He, C. 2012. Expression of multidrug resistance efflux pump gene *norA* is iron responsive in *Staphylococcus aureus*. *J. Bacteriol.* 194(7), 1753–1762.

Dennehy, M., & Williamson, A. 2005. Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. *Vaccine.* 23, 1209–1224.

Dey, A., Verma, A.K., & Chatterji, D. 2010. Role of an RNA polymerase interacting protein, MsRbpA, from *Mycobacterium smegmatis* in phenotypic tolerance to rifampicin. *Microbiol-UK*. 156, 873–883.

Doran, J.L., Pang, Y., Mdluli, K.E., Moran, A.J., Victor, T.C., Stokes, R.W., Mahenthiralingam, E., Kreiswirth, B.N., Butt, J.L., Baron, G.S., Treit, J.D., Kerr, V.J., Van Helden, P.D., Roberts, M.C., & Nano, F.E. 1997. *Mycobacterium tuberculosis efpA* encodes an efflux protein of the QacA transporter family. *Clin. Diagn. Lab. Immunol.* 4(1), 23–42.

Drlica, K., Franco, R.J., & Steck, T.R. 1988. Rifampin and *rpoB* mutations can alter DNA supercoiling in *Escherichia coli. J. Bacteriol.* 170(10), 4983–4985.

Duque, E., Segura, A., Mosqueda, G., & Ramos, J.L. 2001. Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. *Mol. Microbiol.* 39(4), 1100–1106.

Evans, K., Passador, L., Srikumar, R., Tsang, E., Nezezon, J., & Poole, K. 1998. Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa. J Bacteriol.* 180(20), 5443–5447.

Fournier, B., Aras, R., & Hooper, D.C. 2000. Expression of the multidrug resistance transporter NorA from *Staphylococcus aureus* is modified by a two-component regulatory system. *J. Bacteriol.* 182(3), 664–671.

Gagneux, S. 2009. Fitness cost of drug resistance in *Mycobacterium tuberculosis*. *Clin. Microbiol. Infect.* 15(1), 66–68.

Gao, C., Yang, M., & He, Z. 2012. Characterization of a novel ArsR-like regulator encoded by Rv2034 in *Mycobacterium tuberculosis. PLoS One.* 7(4), 1–10.

Garg, S.K., Alam, M.S., Soni, V., Kishan, K.V.R., & Agrawal, P. 2007. Characterization of *Mycobacterium tuberculosis* WhiB1/Rv3219 as a protein disulfide reductase. *Protein Expres. Purif.* 52, 422–432.

Geiman, D.E., Raghunand, T.R., Agarwal, N., & Bishai, W.R. 2006. Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven *Mycobacterium tuberculosis whiB*-like genes. *Antimicrob. Agents Chemother.* 50, 2836–2841.

Gomez, M., Doukhan, L., Nair, G., & Smith, I. 1998. *sigA* is an essential gene in *Mycobacterium smegmatis*. *Mol. Microbiol.* 29(2), 617–628.

Grkovic, S., Brown, M.H., Roberts, N.J., Paulsen, I.T., & Skurray, R.A. 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. *J. Biol. Chem.* 273(29), 18665–18673.

Grkovic, S., Brown, M.H., & Skurray, R.A. 2001. Transcriptional regulation of multidrug efflux pumps in bacteria. *Cell Dev. Biol.* 12, 225–237.

Grkovic, S., Brown, M.H., & Skurray, R.A. 2002. Regulation of bacterial drug export systems. *Microbiol. Mol. Biol. Rev.* 66(4), 671–701.

Grkovic, S., Hardie, K.M., Brown, M.H., & Skurray, R.A. 2003. Interactions of the QacR multidrug-binding protein with structurally diverse ligands: Implications for the evolution of the binding pocket. *Biochemistry*. 42, 15226–15236.

Gu, R., Su, C., Shi, F., Li, M., McDermott, G., Zhang, Q., & Yu, E.W. 2007. Crystal structure of the transcriptional regulator CmeR from *Campylobacter jejuni*. *J. Mol. Biol.* 372, 583–593.

Guazzaroni, M., Krell, T., Gutiérrez del Arroyo, P., Vélez, M., Jiménez, M., Rivas, G., & Ramos, J.L. 2007. The transcriptional repressor TtgV recognizes a complex operator as a tetramer and induces convex DNA bending. *J. Mol. Biol.* 369, 927–939.

Guazzaroni, M., Terán, W., Zhang, X., Gallegos, M., & Ramos, J.L. 2004. TtgV bound to a complex operator site represses transcription of the promoter for the multidrug and solvent extrusion TtgGHI pump. *J. Bacteriol.* 186(10), 2921–2927.

Gupta, A.K., Chauhan, D.S., Srivastava, K., Das, R., Batra, S., Mittal, M., Goswami, P., Singhal, N., Sharma, V.D., Venkatesan, K., Hasnain, S.E., & Katoch, V.M. 2006. Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. *J. Commun. Dis.* 38(3), 246–254.

Hagman, K.E., & Shafer, W.M. 1995. Transcriptional control of the mtr efflux system of *Neisseria gonorrhoeae. J. Bacteriol.* 177(14), 4162–4165.

Hall-Stoodley, L., & Lappin-Scott, H. 1998. Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum*. *FEMS Microbiol. Lett.* 168, 77–84.

Hernández, A., Maté, M.J., Sánchez-Díaz, P.C., Romero, A., Rojo, F., & Martínez, J.L. 2009. Structural and functional analysis of SmeT, the repressor of the *Stenotrophomonas maltophilia* multidrug efflux pump SmeDEF. *J. Biol. Chem.* 284(21), 14428–14438.

Hernández, A., Ruiz, F.M., Romero, A., & Martinez, J.L. 2011. The binding of triclosan to SmeT, the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in *Stenotrophomonas maltophilia*. *PLoS Pathog.* 7(6), 1–12.

Hetherington, S.V., Watson, A.S., & Patrick, C.C. 1995. Sequence and analysis of the *rpoB* gene of *Mycobacterium smegmatis. Antimicrob. Agents Chemother.* 39(9), 2164–2166.

Hu, H., & Ochi, K. 2001. Novel approach for improving the productivity of antibiotic-producing strains by inducing combined resistant mutations. *Appl. Environ. Microbiol*. 67(4), 1885–1892.

Hu, H., Zhang, Q., & Ochi, K. 2002. Activation of antibiotic biosynthesis by specified mutations in the *rpoB* gene (encoding the RNA polymerase β subunit) of *Streptomyces lividans*. *J. Bacteriol*. 184(14), 3984–3991.

Hu, Y., Morichaud, Z., Chen, S., Leonetti, J., & Brodolin, K. 2012. *Mycobacterium tuberculosis* RbpA protein is a new type of transcriptional activator that stabilizes the σ^A-containing RNA polymerase holoenzyme. *Nucl. Acids Res.* 40(14), 6547–6557.

Huitric, E., Werngren, J., Juréen, P., & Hoffner, S. 2006. Resistance levels and *rpoB* gene mutations among *in vitro*-selected rifampin-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob. Agents Chemother.* 50(8), 2860–2862.

Ioerger, T.R., Feng, Y., Ganesula, K., Chen, X., Dobos, K.M., Fortune, S., Jacobs Jr., W.R., Mizrahi, V., Parish, T., Rubin, E., Sassetti, C., & Sacchettini, J.C. 2010. Variation among genome sequences of H37Rv strains of *Mycobacterium tuberculosis* from multiple laboratories. *J. Bacteriol.* 192(14), 3645–3653.

Jiang, X., Zhang, W., Zhang, Y., Gao, F., Lu, C., Zhang, X., & Wang, H. 2008. Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. *Microb. Drug Resist.* 14(1), 7–11.

Johnson, P.J., Stringer, V.A., & Shafer, W.M. 2011. Off-target gene regulation mediated by transcriptional repressors of antimicrobial efflux pump genes in *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* 55(6), 2559–2565.

Johnson, R., Streicher, E.M., Louw, G.E., Warren, R.M., Van Helden, P.D., Victor, T.C. 2006. Drug resistance in *Mycobacterium tuberculosis. Curr. Issues Mol. Biol.* 8, 97-112.

Kent, P.T., & Kubica, G.P. 1985. *Public health mycobacteriology: A guide for the level III laboratory.* Atlanta, GA: United States of America Department of Health and Human Services, Public Health Service, Centres for Disease Control.

Kim, B., Lee, S., Lyu, M., Kim, S., Bal, G., Kim, S., Chae, G., Kim, E., Cha, C., & Kook, Y. 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase Gene (*rpoB*). *J. Clin. Microbiol.* 37(6), 1714–1720.

Konar, M., Alam, M.S., Arora, C., & Agrawal, P. 2012. A cell division associated protein WhiB2/Rv3260c of *Mycobacterium tuberculosis* H37Rv has properties of a chaperone. *FEBS J.* 279(15), 2781–2792.

Kurodo, T., & Tsuchiya, T. 2009. Multidrug efflux transporters in the MATE family. *Biochimica et Biophysica Acta.* 1794(5), 763–768.

Kvist, M., Hancock, V., & Klemm, P. 2008. Inactivation of efflux pumps abolishes bacterial biofilm formation. *Appl. Environ. Microbiol.* 74(23), 7376–7382.

Kyselková, M., Chroňaková, A., Volná, L., Nemec, J., Ulmann, V., Scharfen, J., & Elhottová, D. 2012. Tetracycline resistance and presence of tetracycline resistance determinants *tet*(V) and *tap* in rapidly growing mycobacteria from agricultural soils and clinical isolates. *Microbes. Environ.* In press. doi10.1264/jsme2.ME12028.

Lamarche, M.G., & Déziel, E. 2011. MexEF-OprN efflux pump exports the *Pseudomonas quinolone* signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS One.* 6(9), 1–12.

Landick, R., Stewart, J., & Lee, D.N. 1990. Amino acid changes in conserved regions of the β-subunit of *Escherichia coli* RNA polymerase alter transcription pausing and termination. *Genes Dev.* 4, 1623–1636.

Lee, E.H., Rouquette-Loughlin, C., Folster, J.P., & Shafer, W.M. 2003. FarR regulates the *farAB*-encoded efflux pump of *Neisseria gonorrhoeae* via an MtrR regulatory mechanism. *J. Bacteriol.* 185(24), 7145–7152.

Lee, L., Chen, Y.J., Kirby, R., Chen, C., & Chen, C.W. 2007. A multidrug efflux system is involved in colony growth in *Streptomyces lividans. Microbiol-UK*. 153, 924–934.

Li, X., Zhang, L., & Nikaido, H. 2004. Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis. Antimicrob. Agents Chemother.* 48(7), 2415–2423.

Lin, J., Akiba, M., Sahin, O., & Zhang, Q. 2005a. CmeR functions as transcriptional repressor for the multidrug efflux pump CmeABC in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 49(3), 1067–1075.

Lin, J., Cagliero, C., Guo B., Barton, Y., Maurel, M., Payot, S., & Zhang, Q. 2005b. Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. *J. Bacteriol.* 187(21), 7417–7424.

Liu, J., Takiff, H.E., & Nikaido, H. 1996. Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. *J. Bacteriol.* 178(13), 3791–3795.

Lomovskaya, O., Lewis, K., & Martin, A. 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. *J. Bacteriol.* 177(9), 2328–2334.

Louw, G.E., Warren, R.M., Gey van Pittius, N.C., Leon, R., Jimenez, A., Hernandez-Pando, R., McEvoy, C.R.E., Grobbelaar, M., Murray, M., Van Helden, P.D., & Victor, T.C. 2011. Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. *Am. J. Respir. Crit. Care Med.* 184, 269–276.

Louw, G.E., Warren, R.M., Gey van Pittius, N.C., McEvoy, C.R.E., Van Helden, P.D., & Victor, T.C. 2009. A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob. Agents Chemother.* 53(8), 3181–3189.

Lucas, C.E., Balthazar, J.T., Hagman, K.E., & Shafer, W.M. 1997. The MtrR repressor binds the DNA sequence between the *mtrR* and *mtrC* genes of *Neisseria gonorrhoeae. J. Bacteriol.* 179(13), 4123–4128.

Luo, N., Sahin, O., Lin, J., Michel, L.O., & Zhang, Q. 2003. *In vivo* selection of *Campylobacter* isolates with high levels of fluoroquinolone resistance associated with *gyrA* mutations and the function of the CmeABC efflux pump. *Antimicrob. Agents Chemother.* 47(1), 390–394.

Luong, T.T., Dunman, P.M., Murphy, E., Projan, S.J., & Lee, C.Y. 2006. Transcription profiling of the *mgrA* regulon in *Staphylococcus aureus*. *J. Bacteriol.* 188(5), 1899–1910.

Luong, T.T., Newell, S.W., & Lee, C.Y. 2003. *mgr*, a novel global regulator in *Staphylococcus aureus*. *J. Bacteriol.* 185(13), 3703–3710.

Luria, S.E., & Delbrück, M. 1943. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*. 28, 491–511.

Machowski, E.E., Dawes, S., & Mizrahi, V. 2005. TB tools to tell the tale–molecular genetic methods for mycobacterial research. *Int. J. Biochem. Cell Biol.* 37, 54–68.

Madania, A., Habous, M., Zarzour, H., Ghoury, I., & Hebbo, B. 2012. Characterization of mutations causing rifampicin and isoniazid resistance of *Mycobacterium tuberculosis* in Syria. *Pol. J. Microbiol.* 61(1), 23–32.

Mah, T.C., & O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9(1), 34–39.

Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R., & Smith, I. 1999. Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 31(2), 715–724.

Mariam, D.H., Mengistu, Y., Hoffner, S.E., & Andersson, D.I. 2004. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis. Antimicrob. Agents Chemother.* 48(4), 1289–1294.

Markham, P.N., LoGuidice, J., & Neyfakh, A.A. 1997. Broad ligand specificity of the transcriptional regulator of the *Bacillus subtilis* multidrug transporter Bmr. *Biochem Biophys. Res. Commun.* 239(1), 269–272.

Martins, A., Machado, L., Costa, S., Cerca, P., Spengler, G., Viveiros, M., & Amaral, L. 2011. Role of calcium in the efflux system of *Escherichia coli*. *Int. J. Antimicrob. Ag.* 37, 410–414.

Martins, A., Spengler, G., Rodrigues, L., Viveiros, M., Ramos, J., Martins, M., Couto, I., Fanning, S., Pagès, J.M., Bolla, J.M. Molnar, J., & Amaral, L. 2009. pH modulation of efflux pump activity of multidrug resistant *Escherichia coli*: Protection during its passage and eventual colonization of the colon. *PLoS One*. 4(8), 1–9.

Maughan, H., Galeano, B., & Nicholson W.L. 2004. Novel *rpoB* mutations conferring rifampin resistance on *Bacillus subtilis*: Global effects on growth, competence, sporulation, and germination. *J. Bacteriol*. 186(8), 2481–2486.

Mima, T., & Schweizer, H.P. 2010. The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides, but is a broad-spectrum drug efflux system. *Antimicrob. Agents Chemother.* 54(8), 3113–3120.

Moeller, R., Vlašić, I., Reitz, G., & Nicholson, W.L. 2012. Role of altered *rpoB* alleles in *Bacillus subtillis* sporulation and spore resistance to heat, hydrogen peroxide, formaldehyde, and glutaraldehyde. 194(9), 759–767.

Morlock, G.P., Plikaytis, B.B., & Crawford, J.T. 2000. Characterization of spontaneous, *in vitro*-selected, rifampin-resistant mutants of *Mycobacterium tuberculosis* strain H37Rv. *Antimicrob. Agents Chemother.* 44(12), 3298–3301.

Morris, R.P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., Ehrt, S., Liu, Y., Heifets, L., Pieters, J., Schoolnik, G., & Thompson, C.J. 2005. Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 102(34), 12200–12205.

Newberry, K.J., Huffman, J.L., Miller, M.C., Vazquez-Laslop, N., Neyfakh, A.A., & Brennan, R.G. 2008. Structures of BmrR-drug complexes reveal a rigid multidrug binding pocket and transcription activation through tyrosine expulsion. *J. Biol. Chem.* 283(39), 26795–26804.

Newell, K.V., Thomas, D.P., Brekasis, D., & Paget, M.S.B. 2006. The RNA polymerase-binding protein RbpA confers basal levels of rifampicin resistance on *Streptomyces coelicolor. Mol. Microbiol.* 60(3), 687–696.

Newton-Foot, M., & Gey van Pittius, N.C. 2012. The complex architecture of mycobacterial promoters. *Tuberculosis.* In press. http://dx.doi.org/10.1016/j.tube.2012.08.003.

Nguyen, K.T., Piastro, K., Gray, T.A., & Derbyshire, K.M. 2010. Mycobacterial biofilms facilitate horizontal DNA transfer between strains of *Mycobacterium smegmatis. J. Bacteriol.* 192(19), 5134–5142.

Nicholson, W.L., & Maughan, H. 2002. The spectrum of spontaneous rifampin resistance mutations in the *rpoB* gene of *Bacillus subtilis* 168 spores differs from that of vegetative cells and resembles that of *Mycobacterium tuberculosis*. *J. Bacteriol*. 184(17), 4936–4940.

Nikaido, H. 1998. Multiple antibiotic resistance and efflux. *Curr. Opin. Microbiol.* 1, 516–523.

Nikolaev, Y.A., & Plakunov, V.K. 2007. Biofilm – “City of microbes” or an analogue of multicellular organisms? *Microbiol.* 76(2), 125–138.

Ohno, H., Koga, H., Kohno, S., Tashiro, T., & Hara, K. 1996. Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tube*rculosis strains isolated in Japan. *Antimicrob. Agents Chemother.* 40(4), 1053–1056.

Ojha, A.K., Baughn, A.D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jacobs Jr., W.R., & Hatfull, G.F. 2008. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol.* 69(1), 164–174.

Ojha, A.K., & Hatfull, G.F. 2012. Biofilms of *Mycobaterium tuberculosis:* New perspectives of an old pathogen. In Cardona, P. (ed.). *Understanding tuberculosis: Deciphering the secret life of the Bacilli*. pp. 181–192. New York: InTech. Available from http://www.intechopen.com/books/understanding-tuberculosis-deciphering-the-secret-life-of-the-bacilli/biofilms-of-mycobacterium-tuberculosis-a-new-perspective-of-an-old-pathogen

Pao, S.S., Paulsen, I.T., & Saier, M.H., Jr. 1998. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev*. 62(1), 1–34.

Perkins, A.E., & Nicholson, W.L. 2008. Uncovering new metabolic capabilities of *Bacillus subtilis* using phenotype profiling of rifampin-resistant *rpoB* mutants. *J. Bacteriol*. 190(3), 807–814.

Piddock, L.J.V., Williams, K.J., & Ricci, V. 2000. Accumulation of rifampicin by *Mycobacterium aurum*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis. J. Antimicrob. Chemother.* 45, 159–165.

Putman, M., Van Veen, H.W., & Konings, W.N. 2000. Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* 64(4), 672–693.

Quan, S., Venter, H., & Dabbs, E.R. 1997. Ribosylative inactivation of rifampin by *Mycobacterium smegmatis* is a principal contributor to its low susceptibility to this antibiotic. *Antimicrob. Agents Chemother.* 41(11), 2456–2460.

Rahmati, S., Yang, S., Davidson, A.L., & Zechiedrich, E.L. 2002. Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA. *Mol. Microbiol.* 43(3), 677–685.

Ramón-García, S., Martín, C., Aínsa, J.A., & De Rossi, E. 2006. Characterization of tetracycline resistance mediated by the efflux pump Tap from *Mycobacterium fortuitum*. *J. Antimicrob. Chemother.* 57, 252–259.

Ramón-García, S., Mick, V., Dainese, E., Martín, C., Thompson, C.J., De Rossi, E., Manganelli, R., & Aínsa, J.A. 2012. Functional and genetic characterization of the tap efflux pump in *Mycobacterium bovis* BCG. *Antimicrob. Agents Chemother.* 56(4), 2074–2083.

Ramos, J.L., Martinez-Bueno, M., Molina-Henares, A.J., Terán, W., Watanabe, K., Zhang, X., Gallegos, M., Brennan, R., & Tobes, R. 2005. The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* 69(2), 326–356.

Rao, M., Liu, H., Yang, M., Zhao, C., & He, Z. 2012. A copper-responsive global repressor regulates expression of diverse membrane-associated transporter and bacterial drug-resistance in mycobateria. *J. Biol. Chem.* In press. doi/10.1074/jbc.M112.383604.

Rojas, A., Segura, A., Guazzaroni, M., Terán, W., Hurtado, A., Gallegos, M., & Ramos, J.L. 2003. *In vivo* and *in vitro* evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. *J. Bacteriol.* 185(16), 4755–4763.

Rouquette, C., Harmon, J.B., & Shafer, W.M. 1999. Induction of the *mtrCDE*-encoded efflux pump system of *Neisseria gonorrhoeae* requires MtrA, an AraC-like protein. *Mol. Microbiol.* 33(3), 651–658.

Saier, M.H., Jr, Paulsen, I.T., Sliwinski, M.K., Pao, S.S., Skurray, R.A., & Nikaido, H. 1998. Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *FASEB J.* 12(3), 265–274.

Seoane, A.S., & Levy, S.B. 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance *(mar)* operon in *Escherichia coli*. *J. Bacteriol.* 177(12), 3414–3419.

Sharma, S., Kumar, M., Sharma, S., Nargotra, A., Koul, S., & Khan, I.A. 2010. Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of *Mycobacterium tuberculosis. J. Antimicrob. Chemother.* 65(8), 1694–1701.

Siddiqi, N., Das, R., Pathak, N., Banerjee, S., Ahmed, N., Katoch, V.M., & Hasnain, S.E. 2004. *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. *Infection.* 32(2), 109–111.

Singh, A., Crossman, D.K., Mai, D., Guidry, L., Voskuil, M.I., Renfrow, M.B., & Steyn, A.J.C. 2009. *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. *PLoS Pathog.* 5(8), 1–16.

Singh, M., Jadaun, G.P.S., Srivastava, R.K., Chauhan, V., Mishra, R., Gupta, K., Nair, S., Chauhan, D.S., Sharma, V.D., Venkatesan, K., & Katoch, V.M. 2011. Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant *Mycobacterium tuberculosis* isolates. *Indian J. Med. Res.* 133, 535–540.

Snapper, S.B., Melton, R.E., Mustafa, S., Kieser, T., & Jacobs, Jr, W.R. 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol. Microbiol*. 4(11), 1911–1919.

Srivastava, S., Musuka, S., Sherman, C., Meek, C., Leff, R., & Gumbo, T. 2010. Efflux-pump derived multiple drug resistance to ethambutol monotherapy in *Mycobacterium tuberculosis* and ethambutol pharmacokinetics-pharmacodynamics. *J. Infect. Dis.* 201(8), 1225–1231.

Stewart, P.S., & Costerton, W.J. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet.* 358, 135–138.

Stover, C.K., De La Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs Jr., W.R., & Bloom, B.R. 1991. New use of BCG for recombinant vaccines. *Nature.* 351, 456–460.

Su, C., Rutherford, D.J., & Yu, E.W. 2007. Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 361, 85–90.

Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston, M.J., Matter, L., Schopfer, K., & Bodmer, T. 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis. The Lancet.* 341, 647–650.

Terán, W., Felipe, A., Segura, A., Rojas, A., Ramos, J., & Gallegos, M. 2003. Antibiotic-dependent induction of *Pseudomonas putida* DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. *Antimicrob. Agents Chemother.* 47(10), 3067–3072.

Timm, J., Lim, E.M., & Gicquel, B. 1994. *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusions to *lacZ*: the pJEM series. *J. Bacteriol.* 176(21), 6749–6753.

Tracevska, T., Jansone, I., Broka, L., Marga, O., & Baumanis, V. 2002. Mutations in the *rpoB* and *katG* genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. *J. Clin. Microbiol.* 40(10), 3789–3792.

Truong-Bolduc, Q.C., Dunman, P.M., Strahilevitz, J., Projan, S.J., & Hooper, D.C. 2005. MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. *J. Bacteriol.* 187(7), 2395–2405.

Truong-Bolduc, Q.C., Zhang, X., & Hooper, D.C. 2003. Characterization of NorR protein, a multifunctional regulator of *norA* expression in *Staphylococcus aureus*. *J. Bacteriol*. 185(10), 3127–3138.

Van Bambeke, F., Balzi, E., & Tulkens, P.M. 2000. Antibiotic efflux pumps. *Biochem. Pharmacol.* 60(4), 457–470.

Villellas, C. 2012. Personal communication. EMBO conference: Tuberculosis 2012: Biology, pathogenesis, intervention strategies. 11–15 September. Paris, France: Institut Pasteur.

Wang, R., Prince, J.T., & Marcotte, E.M. 2005. Mass spectrometry of the *M. smegmatis* proteome: Protein expression levels correlate with function, operons, and codon bias. *Genome Res.* 15, 1118–1126.

Warner, D.M., Folster, J.P., Shafer, W.M., & Jerse, A.E. 2007. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the *in vivo* fitness of *Neisseria gonorrhoeae. J. Infect. Dis.* 196, 1804–1812.

World Health Organization. 2012. *Global tuberculosis report 2012.* pp. 8&42. Geneva: WHO Press.

Xiong, A., Gottman, A., Park, C., Baetens, M., Pandza, S., Matin, A. 2000. The EmrR protein represses the *Escherichia coli emrRAB* multidrug resistance operon by directly binding to its promoter region. *Antimicrob. Agents Chemother.* 44(10), 2905–2907.

Yamazaki, Y., Danelishvili, L., Wu, M., Hidaka, E., Katsuyama, T., Stang, B., Petrofsky, M., Bildfell, R., & Bermudez, L.E. 2006. The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. *Cell. Microbiol.* 8(5), 806–814.

Yanofsky, C., & Horn, V. 1981. Rifampin resistance mutations that alter the efficiency of transcription termination at the tryptophan operon attenuator. *J. Bacteriol.* 145(3), 1334–1341.

Zaczek, A., Brzostek, A., Augustynowicz-Kopec, E., Zwolska, Z., & Dziadek, J. 2009. Genetic evaluation of relationship between mutations in *rpoB* and resistance of *Mycobacterium tuberculosis* to rifampin. *BMC Microbiol.* 9, 10–17.

Zhang, L., & Mah, T. 2008. Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J. Bacteriol.* 190(13), 4447–4452.

Zheleznova, E.E., Markham, P.N., Neyfakh, A.A., & Brennan, R.G. 1999. Structural basis of multidrug recognition by BmrR, a transcription activator of multidrug transporter. *Cell*. 96, 353–362.

Zhou, Y.N., & Jin, D.J. 1997. RNA polymerase β mutations have reduced σ^{70} synthesis leading to a hyper-temperature-sensitive phenotype of a σ^{70} mutant. *J. Bacteriol.* 179(13), 4292–4298.

ADDENDUM A

Determining the position of *Rv1258c* transcriptional start site using 5' RACE: alignment results

The position of the transcriptional start site was determined by doing 5' RACE using gene specific primers for the *lacZ* gene in the pJEM15promo vector and sequencing the product obtained. The alignment of the 5' RACE product to part of the pJEM15promo vector sequence is shown in Fig A1. The position of the transcriptional start site would be present immediately downstream of the polyT-tail of the oligo d(T)-anchor primer (Fig 4.1). The oligo d(T)-anchor primer and RACE2 SP3 primer are indicated in green (Fig A1; Table 3.4). The position of the transcriptional start of *Rv1258c* site is indicated in red (Fig A1) Transcriptional starts from an adenine (A) residue. The region from the immediately upstream of the start codon of *Rv1258c* up to the transcriptional start site (six nucleotides) is indicated in light blue (ATATTC) (Fig A1). The *Asp*718 cutting site (GGTACC) which is present in the pRv1258cpF primer used for the initial amplification of the *Rv1258c* gene (Table 3.4), is indicated in purple (Fig A1) and present immediately upstream of the *Rv1258c* promoter region as expected. The start codon of *lacZ* is indicated in orange and the *lacZ* fusion protein start codon in pink (Fig A1).

```
                                 ....|....| ....|....| ....|....| ....|....|
                                     5          15         25         35
pJEM15promo vector sequence      ---------- ---------- ---------- ----------
5' RACE product                  GACCACGCGT ATCGATGTCG ACTTTTTTTT TTTTTTYKT

                                 ....|....| ....|....| ....|....| ....|....|
                                     45         55         65         75
pJEM15promo vector sequence      ---------- ---------- ---ATATTCG GTACCAAGCT
5' RACE product                  TTTYTYTYYT TTTTTKTTTT TTSATATTCG GTACCAATCT

                                 ....|....| ....|....| ....|....| ....|....|
                                     85         95         105        115
pJEM15promo vector sequence      TGATCCGATA ACACAGGAAC AGATCTATGG TTCGTGCAAA
5' RACE product                  TGATCCGAYW ACACAGGAAC AGATCTATGG WTCTYGCWAA

                                 ....|....| ....|....| ....|....| ....|....|
                                    125        135        145        155
pJEM15promo vector sequence      CAAACGCAAC GAGGCTCTAC GAATCGGAAG CTTCGATCCC
5' RACE product                  CAAACGCAAC GAGGCTCTAC GAATCKGAAG CTTCCATCCC

                                 ....|....| ....|....| ....|....| ..
                                    165        175        185
pJEM15promo vector sequence      GTCGTTTTAC AACGTCGTGA CTGGGAAAAC CC
5' RACE product                  GTC------- ---------- ---------- --
```

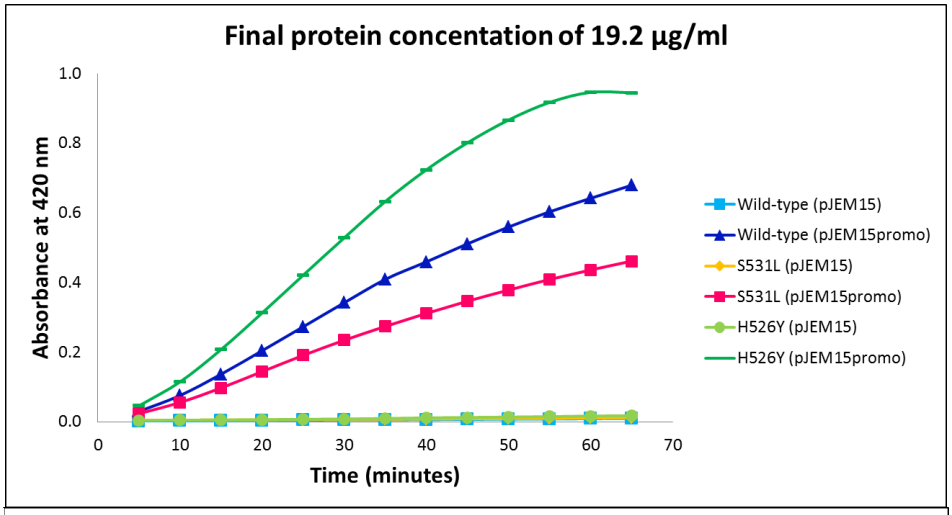
Figure A1. Alignment of the 5' RACE product with pJEM15promo vector sequence.

ADDENDUM B

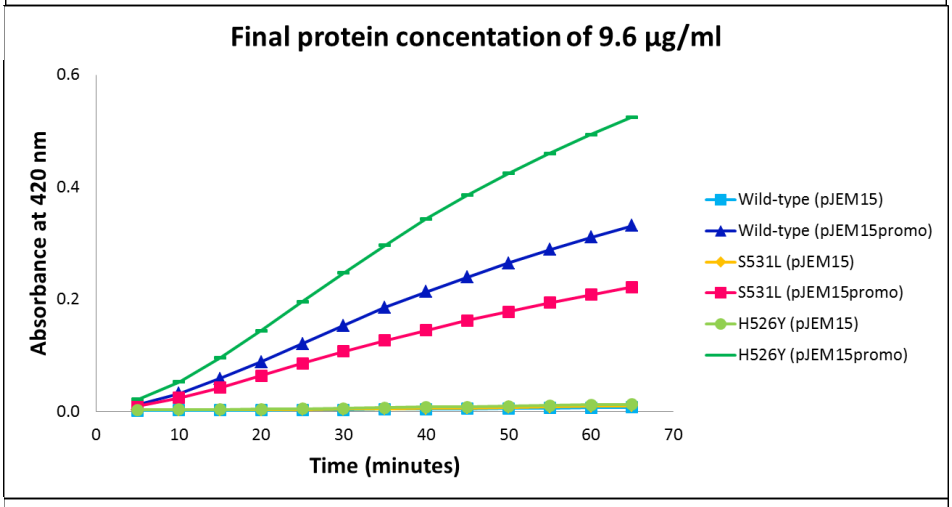
Determining the protein concentration to be used for calculating the β-galactosidase activity

The kinetic curve for *M. smegmatis* mc^2155 *rpoB* H526Y (pJEM15promo) at a protein concentration of 19.2 µg/ml starts to flatten out 50 min into the enzyme reaction and reaches saturation at 55 min (Fig B.1A). The time points from 50 min onward for the 19.2 ng/µl protein concentration could thus not be used for the determining the promoter activity. When looking at the kinetic curve for the 4.8 ng/µl protein concentration, the readings at this protein concentration were still very low (Fig B.1C). The 9.6 µg/ml protein concentration kinetic curve was therefore chosen and the time point of 35 min as the enzyme reaction was not saturated yet at this time point (Fig B.1B). The same principal was used for the *M. smegmatis* strains transformed with the pMV306 integrating vectors and the protein concentration of 153.6 µg/ml and 35 min exposure of the enzyme (β-galactosidase) to the substrate ONPG was chosen as the appropriate protein concentration and time point for calculating the β-galactosidase activity (in Miller units) (kinetic curves not shown). Upon examining kinetic curves for *M. tuberculosis*, a protein concentration of 87.5 µg/ml was chosen for calculating the β-galactosidase value for the wild-type and S531L mutant and 43.75 µg/ml for the H526Y mutant (Fig B.2). An exposure time of 35 min was again used.

A



B



C

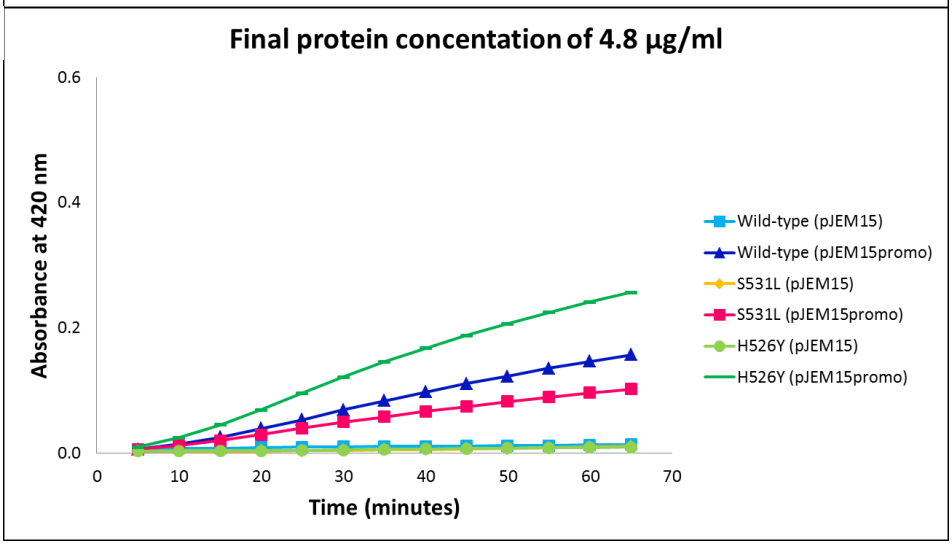
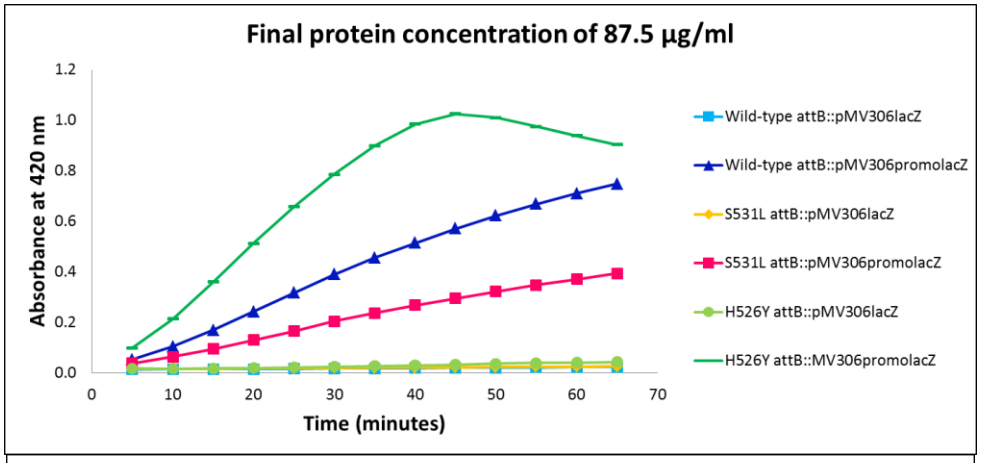


Figure B1. Kinetic curves for β-galactosidase assays in pJEM15 transformed *M. smegmatis* over time exposed to the substrate ONPG at a WCL concentration of (A) 19.2 µg/ml, (B) 9.6 µg/ml and (C) 4.8 µg/ml.

A



B

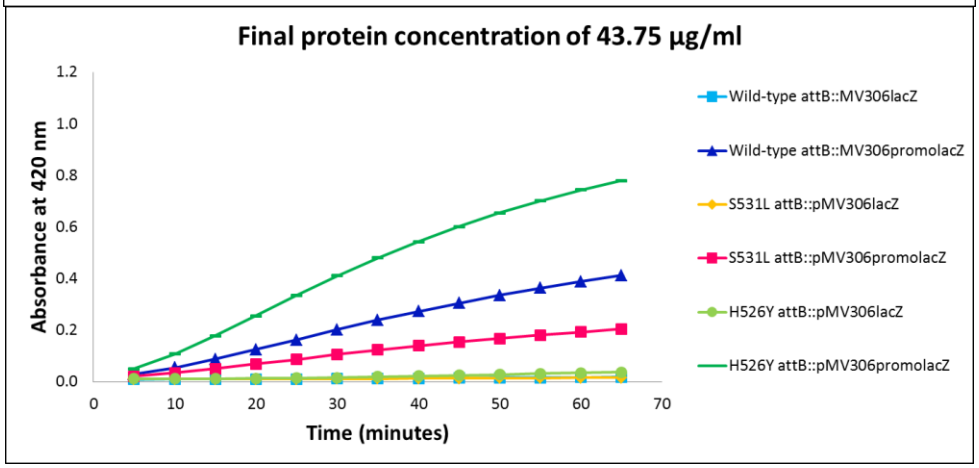


Figure B2. Kinetic curves of β-galactosidase over time exposed to the substrate ONPG at a WCL concentration of (A) 87.5 µg/ml and (B) 43.75 µg/ml.

ADDENDUM C

Calculation of genomic DNA concentration to use for qRT-PCR

The following calculations were done in order to determine the concentration of genomic DNA that represents a final concentration of 10^7 *M. tuberculosis* genomes per 2 µl. This DNA would be used for the generation of a standard curve during subsequent qRT-PCR experiments.

The *M. tuberculosis* genome size is 4,411,529 bp. The average weight of a bp is 650 Dalton.

The molecular weight of one *M. tuberculosis* genome is thus $4{,}411{,}529 \text{ bp} \times 650 \text{ daltons} = 2{,}867{,}493{,}850 \text{ g/mole}.$

This means that 2.8×10^9 g genomic DNA $= 6.022 \times 10^{23}$genomes

Thus 1 µg of genomic DNA = 2.151×10^8 genomes

Therefore the weight of each genome is:

$1 \text{ µg}/2.151 \times 10^8 \text{ genomes} = 4.649 \times 10^{-9} \text{ µg/genome}$

A concentration of 10^7 genomes per 2 µl was needed for the qRT-PCR experiment.

$4.649 \times 10^{-9} \text{ µg/genome} = 4.649 \times 10^{-4} \text{µg}/10^5 \text{ genomes}$

Thus, 46.49 µg/ml in a total volume of 1 000 µl = 10^7genome/µl

A concentration of 23.25 µg/ml = 10^7 genomes/2 µl.

The genomic DNA was diluted to 23.25 µg/ml and this was aliquoted and used for preparation of the dilution range which was used to generate a standard curve.