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UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

**EVALUATION OF A REVERSE TETRACYCLINE
INDUCIBLE SYSTEM IN RECOMBINANT BCG TO
IMPROVE STABILITY AND IMMUNOGENICITY**

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Thesis presented for the Doctor of Philosophy in the Division of Medical Virology, Faculty of Health Sciences, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town

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DECLARATION

The study described in this thesis was performed in the Division of Medical Virology, Department of Clinical Laboratory Sciences of the University of Cape Town, under the supervision and guidance of Doctor Ros Chapman and Professor Anna-lise Williamson. This is my own work using my own words. Each significant contribution to, and quotation in, this thesis from the work, or works, of others has been referenced. Any assistance provided by other people has also been acknowledged. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

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September 2011

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LIST OF ABBREVIATIONS

2YT	2 x yeast tryptone	i.n.	intranasal
Ab	Antibodies	i.p.	intraperitoneal
ADC	Albumin Dextrose Complex	i.r.	intrarectal
AIDS	Acquired Immunodeficiency Syndrome	i.v.	Intravenous
Ami	Acetamidase	Kan	Kanamycin
Amp^R	Ampicillin resistance	Kan^R	Kanamycin resistance
APC	antigen presenting cells	Kb	Kilobase
ARV	Antiretroviral	kD	KiloDalton
ATc	Anhydrous Tetracycline	λ	Lambda
BCG	Bacille Calmette-Guérin	ln	natural logarithm
β-gal	β -galactosidase	log	logarithmic
Bp	base pair	M	Molar
BSA	Bovine Serum Albumin	mM	milli Molar
CBA	Cytometric Bead Array	MIC	minimum inhibitory concentration
cfu	colony forming units	M7H9	Middlebrook 7H9
ConA	Concavalin A	M7H10	Middlebrook 7H10
CRPV	Cottontail Rabbit Papillomavirus	mg	milligram
CTL	Cytotoxic T lymphocyte	MHC	Major histocompatibility complex
DMSO	dimethyl sulphoxide	ml	millilitre
DNA	Deoxyribonucleic acid	MTCT	mother-to-child transmission
Dox	Doxycycline	MVA	Modified Vaccinia virus Ankara
DTH	Delayed-Type Hypersensitivity	N/A	Not applicable
ELISpot	Enzyme Linked Immunospot	Nab	Neutralizing antibodies
EPI	Expanded Programme on Immunization	ng	nanogram
EtBr:	ethidium bromide	nm	Nanometre
FACS	Fluorescence-activated cell sorting	NTM	Non-Tuberculous Mycobacteria
GFP	Green Fluorescent Protein	OADC	Oleic-Acid Albumin Dextrose Complex
HIV	Human Immunodeficiency Virus	°C	Degree Celsius
HLA	Human Leukocyte Antigen	OM	outer membrane
HPLC	High Performance Liquid Chromatography	OD	optical density
HPV	Human Papilloma Virus	ORF	open reading frame
HRP	Horseradish peroxidase	Ori	origin of replication
hsp	heat shock protein	oriE	<i>E. coli</i> origin of replication
HTH	α -helix-turn- α -helix	oriM	Mycobacterial origin of replication
i.d	intradermal	PBS	phosphate-buffered saline
IFN-γ	gamma interferon	pfu	plaque forming unit
IL	Interleukin	Pip	Pristinamycin
i.m.	intramuscular	PPD	Purified Protein Derivative
		r	recombinant

RBC	red blood cells	Tc	Tetracycline
rBCG	recombinant <i>M. bovis</i> BCG $\Delta panCD$	Tet	Tetracycline-inducible
RD	regions of difference	tetO	operator elements
rfu	relative fluorescence unit	tetO₁	Tetracycline operator sequence 1
revTetR	reverse Tetracycline	tetO₂	Tetracycline operator sequence 2
RNA	Ribonucleic acid	Th1	T helper type 1
rpm	revolutions per minute	Th2	T helper type 2
RPP	ribosomal protection protein	TNF	Tumor Necrosis Factor
RT	Reverse Transcriptase	Tra	Transfer
s.c.	subcutaneous	TSAP	shrimp alkaline phosphatase
SCID	severe combined immunodeficient	TSB	Trypiti case soy broth
SD	standard deviation	TLX	Tyloxapol
SDS-	Sodium Dodecyl Sulphate	µl	microlitre
PAGE	Polyacrylamide Gel Electrophoresis		
SV5	V protein of simian virus 5	UCT	University of Cape Town
sfu	spot forming units	UV	ultraviolet
SHIV	Simian-Human Immunodeficiency Virus	VLP	virus-like particles
SIV	Simian Immunodeficiency Virus	WB	Western blotting
TB	Tuberculosis	WHO	World Health Organization

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ABSTRACT

The need for a prophylactic vaccine against HIV remains a global priority as the pandemic continues to expand. An effective HIV vaccine will need to be able to target multiple HIV subtypes or recombinant forms, be safe enough to administer to infants and immunocompromised people and be affordable to guarantee supply in developing countries. Due to its well established safety record, potency and low production cost, *Mycobacterium bovis* Bacille Calmette Guérin (BCG) has been considered as a HIV vaccine vector. BCG expressing HIV proteins under the control of strong promoters have been shown to be unstable. One of the ways of controlling expression in bacteria is through the use of a Tet repressor which is able to bind *tet* operator sequences (*tetO*) only in the presence of the analogue anhydrotetracycline (ATc). This system has been widely used for conditional control of gene expression to determine gene function. The application of this system in rBCG vaccine design is completely novel, and should significantly alleviate the problems associated with instability. The aim of this study is to utilise the tetracycline dependent gene regulation system to down-regulate recombinant antigen expression in BCG during the expansion of seed stocks and up-regulate expression prior to or post vaccination.

The design was based on constitutively expressing the DNA binding repressor protein, TetRr1.12, such that in the presence of ATc, the TetR-ATc complex binds *tet* operator sites placed within a promoter upstream of the recombinant antigen, causing a shutdown of recombinant antigen expression during *in vitro* growth. A series of the episomal TetRr1.12 regulatory systems containing different combinations of *tet* operator sequences (*tetO*₁ and/or *tetO*₂ flanking the -10 region of the *P_{smyc}* promoter) and the *tetRr1.12* repressor gene in different orientations were constructed. The systems were initially evaluated for expression and stability using GFP as a model antigen and then with HIV Gag and reverse transcriptase (RT) as antigens. Two stable recombinant *M. bovis* BCG expressing HIV-1 RT were selected to evaluate the immunogenicity of an rBCG-HIV vaccine using the tetracycline regulated system. The rBCG were administered to mice as a single vaccination in a prime-boost combination with a Modified Vaccinia virus Ankara (MVA) vaccine boost expressing HIV-1 Gag, RT, Tat, Nef and gp150. The frequency of antigen specific cells induced in mice after vaccination were evaluated using an IFN- γ ELISPOT assay and a cytokine bead array assay was used to quantify a spectrum of cytokines released into the culture supernatant during stimulation with RT specific peptides.

The minimum dose of ATc required to achieve repression of GFP expression was determined in *M. smegmatis* and BCG. All the TetRr1.12 systems responded to ATc as GFP expression was repressed in the presence of ATc and induced upon its removal. A minimum ATc dose of 100 - 250 ng/ml achieved a 2.5-fold GFP repression in *M. smegmatis* and 2 µg/ml achieved a 2-fold repression in BCG $\Delta panCD$. The induction upon ATc removal was poor in *M. smegmatis* as none of the recombinants showed maximal induction, whereas rBCG $\Delta panCD$ showed complete induction of GFP.

In the presence of ATc all the recombinant mycobacteria were genetically stable for over ± 42 culture generations. However, in the absence of ATc GFP was over-expressed as depicted by high fluorescence, a reduction in colony size and genetically unstable recombinants, in *M. smegmatis* after 42 generations, <56% recombinants harbouring the *tetRr1.12* gene in the same orientation as the *gfp* gene and <2% recombinants harbouring the *tetRr1.12* gene in the opposite orientation to the *gfp* gene were genetically intact. In BCG $\Delta panCD$ after 49 generations, 76 - 81% recombinants harbouring the *tetRr1.12* gene in the same orientation as the *gfp* gene and 57 - 74% recombinants harbouring the *tetRr1.12* gene in the opposite orientation to the *gfp* gene remained genetically intact.

The recombinants containing the *tetRr1.12* gene in the same orientation as the GFP, pPM3r2.1 and pPM4r2.1 were the most stable and thus were selected to generate the episomal HIV-TetRr1.12 plasmids, pPM3RT, pPM3Gag, pPM4RT and pPM4Gag by replacing the *gfp* gene in the plasmids with the HIV-1 subtype C *rt* and *gag* genes. Included in the study were the episomal multicopy HIV-TetRr1.7 systems, pRC3RT and pRC3Gag that utilise the TetRr1.7 repressor for regulation. The pRC3RT and pRC3Gag contain the *tetRr1.7* gene orientated in the same direction as the HIV-1 *rt* and *gag* genes, respectively. Stability assessment revealed that rBCG $\Delta panCD$ [HIV-TetR] harbouring the *gag* gene were unstable as a 1170 bp fragment containing p24, p6 and the V3-SV5 epitope tag was deleted. Two of recombinants harbouring the *rt* gene, rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] were stable, but the rBCG $\Delta panCD$ [pPM4RT] lost a 1452 bp fragment containing the *rt* gene and the V3-SV5 epitope tag. The genetically stable systems were used to generate rBCG HIV-1 subtype C vaccines and the immunogenicity assessed in BALB/c mice.

A prime with the rBCG $\Delta panCD$ vaccines via the intraperitoneal route and a boost with an MVA elicited antigen-specific intracellular IFN- γ expression in the mice splenocytes in response to

stimulation with HIV-1 peptides. Compared to the responses elicited by the control, rBCG $\Delta panCD$ [pCONEPI] prime and MVA-C boost, the responses elicited by rBCG $\Delta panCD$ vaccines and MVA-C boost were higher, the responses to the V3-CTL CD8 peptide were 4.2-fold higher, the responses to the RT-CD8 peptide were 5.6-fold and the responses to the RT-CD4 were 2.4-fold higher. Removing ATc from the vaccines prior to vaccination did not alter the immune response significantly as rBCG vaccines that contained ATc elicited comparable levels of immune responses as those without ATc. Additionally, the spectrums of HIV-specific cytokines released from the splenocytes were mainly IFN- γ and TNF- α .

A stable rBCG vaccine expressing HIV-1 RT antigen has been developed, where antigen expression can be down-regulated *in vitro* to improve vector stability and up-regulated *in vivo* to induce strong immune responses. These results indicate that we overcame the challenges of low antigen expression caused by genetic instability that can lead to the weak immunogenicity usually associated with expression of viral antigens in BCG.

CHAPTER 1: A REVIEW ON BCG AS A TB AND A LIVE BACTERIAL VACCINE

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1.1. MYCOBACTERIUM BOVIS BACILLE CALMETTE GUÉRIN: A TB VACCINE

Tuberculosis (TB), a chronic infectious disease caused by *Mycobacterium tuberculosis* accounts for over 2 million deaths per year ^{1, 2}. The TB vaccine, Bacille Calmette Guérin (BCG), was developed by a French bacteriologist, Albert Calmette and his colleague Camille Guérin at the Institut de Lille in France in 1908 ^{3, 4}. They isolated a virulent strain of the bovine tubercle bacillus *Mycobacterium bovis* from a cow with TB and attenuated it by repeatedly subculturing it on glycerinated bile-potato medium through more than 200 serial passages ^{5, 6}. It took over 13 years for the strain to completely lose virulence ^{4, 7}. The attenuated *M. bovis* strain was evaluated intensively *in vitro* and in animal models between 1908 and 1921 and there was no evidence of reversion to virulence, implying that the attenuation resulted from very stable deletions and mutations ^{6, 8, 9}.

1.1.1. THE GLOBAL USE OF BCG AS A TB VACCINE

The first BCG vaccine was administered by Weil Halle in Paris in 1921 ¹⁰. It was given orally to a newborn whose mother had died soon after giving birth from TB ^{5, 11}. The infant was given 3 doses of 2 mg (8 x 10⁷ bacillary elements per dose) at 3, 5 and 7 days after birth ¹⁰. The child was immunized against TB throughout his life span ¹².

BCG was officially adopted by the Health Committee of League of Nations as a TB vaccine in 1928, and introduced into the World Health Organization (WHO) and Expanded Programme on Immunization (EPI) in 1974 ^{7, 13-15}. Over the years the BCG vaccine has been given to millions of infants, and it has been followed by the reports of mortality rate reduction ⁵.

The practice of intradermal (i.d.) administration was introduced in 1927, the percutaneous route was introduced in 1931, and the oral route was discontinued in 1973 ^{5, 14, 16}. To date BCG is used in TB control programs and it is recommended by the WHO for all infants under the age of 1 year unless they are infected with HIV ¹⁷⁻²⁰. Every year, about 80% of the world's infant population receive BCG vaccination, principally in developing countries where TB is highly endemic (Figure 1.1) ^{11, 14, 21}. In South Africa the BCG vaccine has been administered as routine neonatal vaccination since 1974 ²². It has displayed a high protective efficacy when given to

newborns against life-threatening forms of childhood TB, such as TB meningitis and miliary TB²³⁻²⁶. Furthermore, Fine *et al.*, (1999)²⁷ reported that BCG vaccination also protects against leprosy.

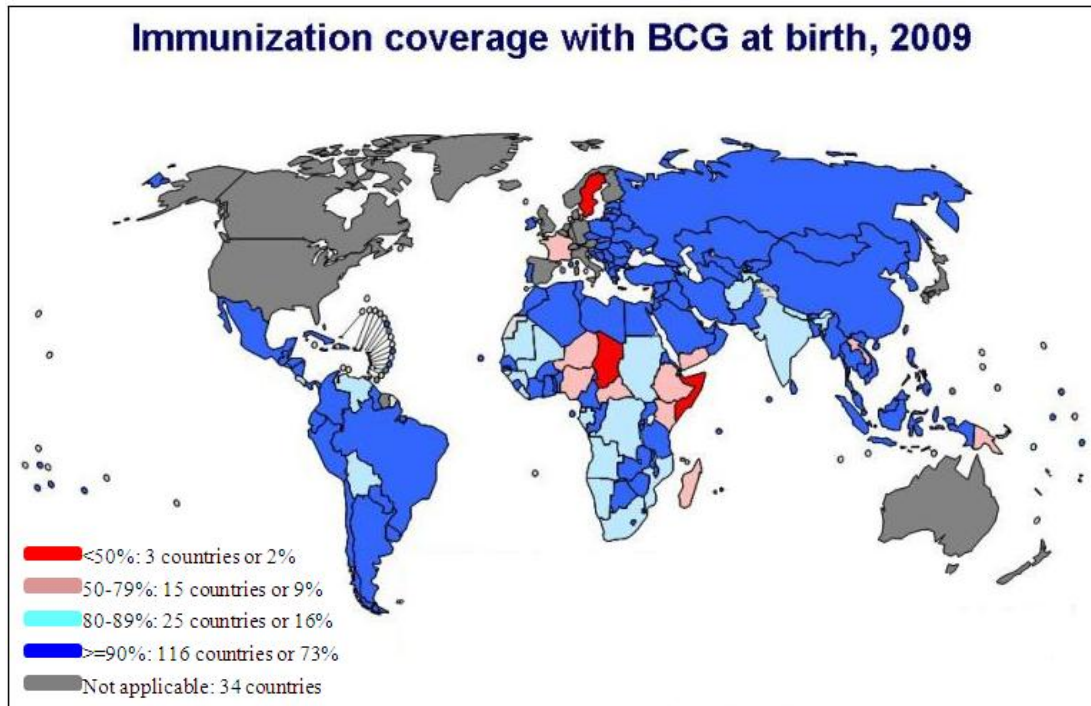


Figure 1.1: WHO/UNICEF global BCG immunization coverage at 88% in 2009, estimated by 193 WHO Member States 27 July 2010. Taken from, http://www.who.int/immunization_monitoring/diseases/tuberculosis/en/index.html.

1.1.2. CHALLENGES FACING THE USE OF THE BCG AS A TB VACCINE

Despite the numerous advantages that make BCG an attractive vaccine, there are a few negative aspects that indicate that the vaccine could be improved. BCG has variable protective efficacy. In addition, cases of disseminated BCG disease have been reported in immunocompromised individuals after BCG vaccination^{5, 28-31}. Furthermore BCG sensitizes for the tuberculin skin test (a test used to determine if an individual or an animal has developed an immune response to the bacterium that causes TB) and thus compromises the use of this test in humans and animals, which is one of the reasons why BCG is not a routine vaccine in the United States³².

1.1.2.1. Variable efficacy of the BCG vaccine

BCG vaccine efficacy has been assessed in numerous clinical trials and the most controversial element of the vaccine is its variable efficacy in preventing TB, which ranges from 0 - 80% with an average protection of 50%^{14, 26, 33-36}. While BCG vaccine protects against childhood TB, this protective efficacy wanes over an estimated period of 10 - 15 years and consequently the vaccine often fails to protect adults from pulmonary TB and other mycobacterial diseases^{24, 37, 38}. The lowest BCG vaccine efficacy is found in the tropical regions of the world, such as Sub-Saharan Africa and South-East Asia^{35, 39, 40}.

There are a number of proposed reasons for this discrepancy in protective efficacy of BCG against TB, most of which lack strong clinical support^{15, 38, 40-42}. These reasons include genetic variations in BCG strains, different preparation methods and administration routes and doses of the vaccine^{3, 43-45}, occurrence of environmental non-tuberculous mycobacteria (NTM) and other mycobacterial infections^{23, 38, 46}, genetic variation, age and nutrition status and socioeconomic conditions of the hosts^{24, 34, 47} and the inadequacy of the antigenic repertoire of *M. bovis* BCG⁴⁸. Furthermore a few studies have reported that the waning protective immunity against TB could be attributed to the waning memory immunity induced by BCG, which is robust during childhood after vaccination and then weakens as the individual approaches adulthood^{37, 49, 50}. Aydinlioglu *et al.*, (1993)³⁷ assessed the immunity of 219 healthy newborns from 3 - 12 months after BCG vaccination. They observed a decline in the immunity to the tuberculin, as an 87% reactivity was observed at 3 months and a 61% at 12 months.

a. Genetic variation of BCG strains

Pasteur is the original attenuated *M. bovis* strain^{3, 8, 41}. This strain has been propagated many times in a large number of laboratories since 1921. Consequently deletions, duplications and single nucleotide mutations have occurred in the genome of the strain^{8, 51}. Therefore BCG strains are genetically and phenotypically dissimilar. At present the five main *M. bovis* BCG strains that are produced and distributed worldwide, are Tokyo, Connaught, Russian, Danish and Moreau^{3, 43, 52}. These strains vary morphologically, biochemically and in protein expression within the hosts, and thus display different immunogenicity^{3, 41, 43, 44}.

b. The possible impact of environmental mycobacterial infections on the efficacy of BCG vaccination

One hypothesis for the differing efficacy of BCG vaccination is based on the different prevalence of environmental mycobacteria in different regions. It has been scientifically proven in animal studies that environmental NTM interfere with or inhibit the multiplication of BCG and thus preclude the induction of protective immunity to TB^{23, 34, 53}. Thus in geographical areas where environmental mycobacteria are abundant, such as tropical regions, the BCG vaccine has shown the lowest protective efficacy^{11, 23}. To confirm this hypothesis Black *et al.*, (2001)³⁹ assessed blood samples obtained from individuals who had not been vaccinated with BCG living in northern Malawi, where the BCG vaccine has a very low efficacy. The blood samples showed positive interferon gamma (INF- γ) responses to a variety of mycobacterial antigens, which indicated that these individuals were greatly exposed to environmental mycobacteria.

1.1.2.2. Risks associated with BCG vaccines

Until recently, BCG has been considered to be very safe, now it is contraindicated for immunocompromised, malnourished or asymptomatic HIV-positive infants as these individuals have an increased risks of regional, extra-regional, localized and disseminated BCG disease^{17, 54, 55}. Furthermore, BCG-induced immune reconstitution inflammatory syndrome (IRIS) has been identified in children receiving antiretroviral therapy^{22, 56}. As a result the WHO recommends that the BCG vaccine not be given to HIV-infected, asymptomatic HIV-positive or immunocompromised infants^{20, 22}.

1.1.3. STRATEGIES DESIGNED TO ENHANCE BCG VACCINE EFFICACY AGAINST TB

Despite its variable efficacy and failure to protect against pulmonary disease, BCG is still able to confer protection against TB meningitis and leprosy^{14, 23, 25}. Consequently the vaccine is included in most TB vaccine strategies^{11, 57, 58}. The development of a safe and efficacious BCG vaccine against TB has been an ongoing process for years^{59, 60}. Various strategies have been designed and evaluated in a variety of preclinical experiments using different animal models and some have completed Phase I clinical trials^{18, 55, 61-63}. These strategies include BCG-homologous prime boost immunization regimens^{58, 64, 65}, BCG-heterologous prime-boost immunization regimens^{23, 31, 66-68}, reintroduction of the region of deletion-1 (RD1) locus into BCG^{6, 69-71}, the use

of subunit vaccines^{72, 73}, TB polyprotein vaccines^{40, 74}, DNA vaccines^{75, 76}, the use of adjuvants^{62, 77}, expression of major antigens of *M. tuberculosis* in a separate vaccine vector, such as poxviruses (including modified vaccinia virus ankara (MVA)) and adenoviruses^{58, 68, 78} and the development of auxotrophic vaccines^{18, 55, 79}, which will be reviewed in the following section.

The ability of the BCG vaccine to elicit cell-mediated immunity, particularly INF- γ secreting T-cells, is responsible for protection against TB^{39, 45, 80, 81}. This immunogenicity of the BCG has led to its investigation as a heterologous antigen delivery vehicle (reviewed extensively in the following section). Recombinant BCG engineered to express high levels of immunodominant antigens of *M. tuberculosis* have been constructed against TB^{18, 63, 82-84}. These antigens are conserved amongst all mycobacterial species, exported and very immunodominant in both animals and humans^{18, 58, 63, 78, 85}. These proteins belong to the mycolyl-transferase antigen 85 complex (Ag85A, Ag85B and Ag85C), a group of the major secretory proteins^{58, 85, 86}.

While prime-boost regimens appear to be very promising strategies for the development of an improved TB vaccine given the waning immunity to BCG, various reviews have reported that the heterologous prime boosts are superior to homologous prime boosts in term of BCG efficacy enhancement^{11, 58}. The advantage of heterologous prime boost regimens is that two different vaccines expressing the same antigen are used, and thus any of the vaccine strategies listed above can be and have been used to boost a BCG prime^{30, 58, 66, 77, 87, 88}. Furthermore since in developing countries BCG is administered to all infants, development of an improved BCG boost is a focus of the TB vaccine research. The use of rBCG prime with heterologous boost will be discussed further in Section 1.2.7.2 d.

1.2. THE USE OF BCG AS A HETEROLOGOUS ANTIGEN DELIVERY VEHICLE

Preceding the 1990s there was a lack of information on BCG and its genetic mechanisms as it was not commonly studied due to its slow growth rate and complex cell wall ⁷. The interest in BCG grew after the development of molecular tools and methods that facilitate the manipulation of the strain and allow simple transfer, expression and secretion of heterologous antigens in this strain ⁸⁹⁻⁹². As a result BCG has been researched extensively and its recombinant forms, rBCG expressing heterologous antigens have been constructed and evaluated *in vitro* and *in vivo*.

1.2.1. RECOMBINANT BCG

Recombinant *M. bovis* BCG (rBCG) has been tested as a monovalent vaccine in numerous animal models and human cells to deliver viral, bacterial and parasitic antigens, where it expresses antigens for specific diseases ^{68, 84, 93-96}. In several animal studies rBCG has conferred protection against diseases. In mice Grode *et al.*, (2002)⁹⁷ showed anti-listerial protection induced by BCG expressing the p60 antigen of *Listeria monocytogenes*, which protected the animals against *L. monocytogenes* challenge. Govan *et al.*, (2006)⁹⁸ reported that outbred New Zealand White rabbits were protected against cottontail rabbit papillomavirus (CRPV) challenge following immunization with rBCG expressing the CRPV major capsid protein, L1. Other diseases that have been protected against by rBCG expressing defined antigens include Lyme disease ⁹⁹, pneumococcal infection ¹⁰⁰, malaria ¹⁰¹ and *Bordetella pertussis* ²¹.

In some studies rBCG has prolifically expressed the recombinant antigens and evoked robust immune responses, however only conferred partial or no protective immunity ⁹⁶. Immunization of sheep with rBCG expressing one of the major excreted/secreted *Toxoplasma gondii* antigens, GRA1 lead to the induction of strong GRA1-specific cell-mediated responses, but only partially protected the animals against oocyst challenge infection¹⁰². Other antigens that have been successfully expressed in BCG are the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and simian immunodeficiency virus (SIV) antigens, which will be discussed in detail in the next section. Considering the above studies it is evident that live recombinant BCG vaccines expressing pathogen-derived antigens present potential vaccine candidates for development of future vaccination strategies.

The global use of BCG as a live vaccine vector is due to a number of features. BCG has been found to be reasonably safe as it can be administered orally to infants and it shows a very low incidence of side effects^{102, 103}; it is not affected by maternal antibodies as it can be given at any time after birth^{104, 105}; it is simple and inexpensive to produce^{92, 106, 107}; it is heat-stable¹⁰⁵; and its cell wall components make it a potent adjuvant^{102, 108, 109}. BCG can be genetically manipulated or altered fairly easily to maximise its immunogenicity¹⁰⁵; and it has the ability to express large heterologous genes^{92, 110-112}. BCG is persistent *in vivo* seeing that it survives in the human antigen presenting cells (APC), such as macrophages and dendritic cells for long periods of time and consequently a single inoculum of BCG can elicit long-lasting immunity as the antigen is continuously produced and presented to the immune system^{60, 92, 104, 113, 114}. In addition, rBCG vaccines can elicit either cellular or humoral, or both of these immune responses against heterologous antigens^{91, 93, 109, 115, 116}.

1.2.2. RECOMBINANT BCG EXPRESSING HIV- 1 ANTIGENS

HIV, the causative agent of AIDS has been a global pandemic for almost 30 years¹¹⁷. Millions of HIV infections and deaths have occurred, and an effective vaccine still remains elusive^{118, 119}. There have been a number of major scientific challenges in developing a vaccine for this virus, for instance its genetic diversity as a result of high mutation rate in viral replication, the lack of immune correlates of protection, as well as the genetic and geographical diversity of HIV where prototype viruses have not been identified¹²⁰⁻¹²⁴. Although antiretroviral (ARV) therapy has become increasingly available and has proven successful in the suppression of viral load and disease, access in Africa remains limited due to cost and uncertainty of supply^{125, 126}. As a result, effective, inexpensive HIV vaccines remain our greatest hope of fighting AIDS.

Researchers are aiming to develop a safe, immunogenic and affordable AIDS vaccine that will reduce this pandemic. Several vaccine strategies such as neutralizing antibodies (NAbs); DNA vaccines; prime-boost regimens; subunit vaccines; particle-based vaccines and live vectors have been developed and tested in an attempt to develop an HIV vaccine^{67, 78, 124, 127}.

It has been reported that AIDS is the result of cross-species infections of humans by lentiviruses of primate origins¹²⁸. One example of such infections is SIV. SIV infects different species of nonhuman primates and has been reported to be the cause of AIDS in these species. The

SIV/macaque model is currently the most appropriate animal model seeing that its infectivity and immunogenicity are very similar to those of humans. Consequently rBCG vaccine vectors expressing different HIV and SIV immunogens have been studied in small animal models and nonhuman primates in order to evaluate their immunogenicity, and the major studies are listed in Table 1.1.

One of the first studies performed in an animal model was conducted by Aldovini and Young, (1991)¹⁰⁴ in BALB/C mice, where they reported induction of both antigen-specific cellular and antibody responses after immunization with rBCG expressing a variety of HIV-1 polypeptides. In the subsequent years studies have been conducted to show that rBCG can induce both of these responses to the major proteins of HIV or SIV in different animal models, such as rhesus macaques¹²⁹⁻¹³¹, baboons¹³², guinea pigs^{112, 133} and BALB/c mice^{105, 134}.

1.2.2.1. Neutralizing antibody responses to HIV- 1 antigens

It is hypothesized that virus-neutralization plays a role in controlling virus infection, thus neutralizing antibodies (NAb) are still considered as one of the major effector mechanisms against HIV infection¹³⁵. In some rhesus macaque studies, NAb have completely blocked experimental infections¹³⁶⁻¹³⁸. These responses have been explored in BCG/HIV vaccine studies in an attempt to neutralize different strains in order to reduce initial viral load. Most of these studies aim to induce long lasting antibodies against the envelope glycoprotein^{135, 139}.

In BALB/c mice and guinea pigs Lim *et al.*, (1997)¹⁴⁰ demonstrated induction of Env specific IgG antibodies (Ab) after an oral immunization with rBCG expressing SIV Env, which neutralized growth of virulent SIVmac251 field isolates *in vitro*. Similarly Hiroi *et al.*, (2001)¹³⁵ also induced high titres of Env V3-specific IgG antibodies in the serum of mice vaccinated with rBCG-V3J1 via nasal immunization, which were able to neutralize clinical HIV-1 isolates *in vitro*. Additionally, expressing HIV *env* V3 in BCG Someya *et al.*, (2005)¹²⁷ demonstrated induction of a strong antigen-specific NAb response for 24 weeks in rhesus macaques. Although the response did not provide protection against a pathogenic SHIV challenge, it significantly reduced viral load after a challenge with a non-pathogenic SHIV.

1.2.2.2. Specific T-cell responses to HIV- 1 antigens

Studies have shown that cell-mediated immunity, cytotoxic T lymphocyte (CTL) and Th1-type responses are effective against HIV since these responses prolong disease progression by releasing cytokines that reduce viral load^{141, 142}. It is speculated that CD4⁺ T-cell responses can limit HIV-1 transmission and vigorous CD8⁺ T-cell-mediated anti-HIV-1 responses might help to maintain low viraemia as seen in long-term non-progressors and acute seroconverters¹⁴³⁻¹⁴⁶.

Generally to induce CTLs, researchers generate rBCG vaccines expressing peptides derived from the structural and regulatory proteins such as Env, Gag, Pol and Nef^{129, 133, 147}. Yasutomi *et al.*, (1993)¹²⁹ demonstrated induction of CTLs to Gag in rhesus monkeys immunized intradermally with rBCG-SIV_{mac}gag. Using rBCG-Env V3 (Table 1.1) to immunize guinea pigs and mice, Honda *et al.*, (1995)¹³³ showed induction of CTLs, which played a role in protection of the animals against HIV infection. Winter *et al.*, (1995)¹⁴⁸ demonstrated that CTLs are induced to the central region of Nef in BALB/c mice vaccinated with rBCG expressing SIV_{mac251} *nef* gene. Similarly Lagranderie *et al.*, (1997)⁹³ observed strong systemic and mucosal CTLs to Nef in BALB/c mice following oral immunization with rBCG SIV *nef*.

Sequence analysis have suggested that CTL epitopes conserved across different genetic HIV subtypes and directed against Gag, Env, Pol and RT sequences exist¹⁴⁹⁻¹⁵¹. These epitopes are expected to reduce the occurrence of escape mutants in HIV infected individuals. Therefore another strategy employed to overcome the emergence of escape mutants is the development of polyepitope rBCG vaccine. The polyepitope rBCG would be generated by integrating multiple sequences encoding HIV T and B cell epitopes into a single vector¹³⁹.

Table 1.1: A summary of some immunogenicity studies on SIV and HIV immunogens expressed in BCG as vaccine vector (data modified and updated from Chapman *et al.*, 2010¹⁵²)

HIV/SIV GENE/ANTIGENS EXPRESSED BY BCG	EXPERIMENTAL DESIGN	ANIMAL MODEL	KEY RESULTS	REFERENCES
HIV-1 Env gp120 expressed under the control of <i>hsp60</i> promoter	Single inoculation with 2x10 ⁶ cfu BCG containing integrating vector expressing HIV-1 Env gp120 or model antigens (β -galactosidase or fragment C of tetanus toxin)	BALB/C mice	<ul style="list-style-type: none"> CTL responses (specific lysis) to HIV-1 Env gp120 immunodominant epitope, P18 peptide Induction of humoral responses to model antigens (β-gal and tetanus toxoid) Induction of cellular immune responses (IFN-γ production) in animals vaccinated with rBCG-β-gal, and not with rBCG-gp120. 	Stover <i>et al.</i> , 1991 ¹⁰⁵
HIV-1 gag, pol and env expressed separately under the control of <i>hsp70</i> promoter	Single i.d. or i.v. inoculations with 5x10 ⁶ cfu of rBCG-gag or rBCG-env	BALB/C mice	<ul style="list-style-type: none"> Induction of humoral responses to HIV-1 proteins in all mice inoculated intravenously Induction of CTL responses (specific lysis) in mice inoculated with rBCG-gag; CTL activity attributed to CD8⁺ splenocytes Induction of cellular immune responses (IFN-γ & IL-2 production) in animals vaccinated with rBCG-gag. 	Aldovini and Young, 1991 ¹⁰⁴
SIVmac251 gag expressed under the control of <i>hsp70</i> promoter	Two i.d. vaccinations with rBCG at 4 sites (dose:10 ⁸ cfu) 19 weeks apart	<i>mamu-A*01</i> ⁺ rhesus macaques	<ul style="list-style-type: none"> Immunization elicited MHC class 1-restricted CD8⁺ SIVgag-specific CTL No anti-SIVmac gag antibody response was generated in immunized animals. 	Yasutomi <i>et al.</i> , 1993 ¹²⁹
SIVmac251 <i>nef</i> gene under the control of <i>M. paratuberculosis</i> P_{AN} promoter	Inoculation with 10 ⁷ cfu rBCG(SIV _{mac251} <i>nef</i>) via s.c.	BALB/C mice	<ul style="list-style-type: none"> Proliferative and CTL responses against Nef peptides. 	Winter <i>et al.</i> , 1991 ¹⁴⁸

<p>HIV <i>env</i> V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence NTRKSIHIGPGRAFYATGS which has a neutralization sequence identical to that of HIV-1MN)</p>	<p>Animals were vaccinated once with rBCG by s.c. injection at a dose of 5 mg (~10⁸ cfu; guinea pigs) and 0.1 mg (~3x10⁶; mice).</p>	<p>guinea pigs and mice</p>	<ul style="list-style-type: none"> ▪ DTH responses to Env V3 and PPD were induced in guinea pigs after immunization with rBCG-Env V3 and following systemic passive transfer of spleen cells obtained from rBCG-Env V3-vaccinated guinea pigs ▪ CTL responses were induced in mice by vaccination with rBCG-Env V3 ▪ guinea pig immune antisera had ability to (i) neutralize primary HIV-1 isolates and (ii) block HIV infection in SCID mice. 	<p>Honda <i>et al.</i>, 1995¹³³</p>
<p>SIVmac251 gag expressed under the control <i>hsp70</i> promoter</p>	<p>Two i.d. vaccinations with rBCG at 4 sites (dose:10⁸ cfu) 19 weeks apart, subsequent boosting (13 months after) with SIVmac Gag peptide p11C (dose: 1 mg; i.m.) formulated with a lipid-A-containing liposome in aluminium hydroxide, and pathogenic SIV challenge</p>	<p><i>mamu-A*01</i>⁺ rhesus macaques</p>	<ul style="list-style-type: none"> ▪ Vigorous p11C-specific CTL response generated was greater than those generated by a single modality immunization with p11C peptide-liposome inoculation ▪ No protection was afforded by this CTL response against SIV challenge ▪ Recovered virus after challenge had identical SIV gag demonstrating that the CTL response did not select for viral variant that might escape T-cell immune recognition 	<p>Yasutomi <i>et al.</i>, 1995¹⁵³</p>
<p>SIVmac251 nef expressed under the control of P_{AN} promoter</p>	<p>Oral immunization with rBCG (10⁹ cfu) for 5 consecutive days (total dose of 5x10⁹ cfu)</p>	<p>BALB/c mice</p>	<ul style="list-style-type: none"> ▪ rBCG translocated to oropharyngeal mucosa and intestinal epithelium ▪ Strong systemic and mucosal responses were induced ▪ Specific anti-Nef CTLs demonstrated in intraepithelial CD8⁺ cells 	<p>Lagranderie <i>et al.</i>, 1997⁹³</p>
<p>SIVmac251env gp110 fused to β-lactamase gene and encoding gene under the control of the <i>pBlaF*</i> promoter</p>	<p>inoculation with (dose: 10⁷ cfu) via s.c. immunization</p>	<p>BALB/c mice and guinea pigs</p>	<ul style="list-style-type: none"> ▪ Strong CTL responses and humoral immune responses elicited in both mice and guinea pigs immunized by parenteral routes ▪ The anti-gp110 IgGs produced were able to neutralize <i>in vitro</i> growth of virulent SIVmac251 field isolates ▪ guinea pigs immunized by the oral route produced significant levels of anti-gp110 IgAs in the faeces, demonstrating that rBCG is able to induce local humoral immunity in the intestinal mucosa 	<p>Lim <i>et al.</i>, 1997¹⁴⁰</p>

SIVmac251 Nef protein, and large fragments of the Env and Gag proteins	Immunization via different mucosal routes (oral, aerogenic, nasal, and rectal)	BALB/c mice	<ul style="list-style-type: none"> Local, specific IgA, systemic IgG, strong, specific cytotoxic responses of splenocytes against Nef, Env, and Gag was observed whatever the mucosal route of immunization 	Lagranderie <i>et al.</i> , 1998 ¹¹⁰
SIVmac251 Gag, Pol, truncated Env and Nef (separately subcloned into shuttle vector under regulatory control of the <i>hsp70</i> promoter)	Animals inoculated i.v. with 4 rBCG constructs containing SIV <i>gag</i> , <i>pol</i> , <i>env</i> , and <i>nef</i> combined in a single inoculum (dose: 5-7x10 ⁸ cfu)	rhesus macaques	<ul style="list-style-type: none"> Vaccination elicited SIV-specific IgA and IgG antibody, and weak cellular immune responses, including CTL and helper T cell proliferation to Gag, Pol and Env 	Leung <i>et al.</i> , 2000 ¹³⁰
HIV <i>env</i> V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	Animals inoculated with rBCG either by nasal (10 µg, ~10 ⁶ cfu), oral (100 µg) or s.c. (100 µg) routes, once a week for 3 consecutive weeks	C57BL/6J mice	<ul style="list-style-type: none"> High titres of Env V3-specific IgG antibodies were generated in the serum of nasally-immunized animal, maintained for >12 months, and had ability to neutralize clinical HIV-1 isolates <i>in vitro</i> Env V3-specific IgG-producing cells were detected in mononuclear cells obtained from spleen, nasal cavity and salivary gland 	Hiroi <i>et al.</i> , 2001 ¹³⁵
HIV <i>env</i> V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	rBCG-HIVEnv inoculated via either a single (i.d., i.n. and i.r.) or combination (i.r./i.d. and i.r./s.c.) routes. (i.r. = 80 mg of vaccine once a week for 2-4 consecutive weeks; s.c. and i.d. = 1.0 and 0.1 mg once respectively; i.n. = 10 µg 4 times weekly for 3 weeks)	guinea pigs	<ul style="list-style-type: none"> DTH responses to both PPD and Env-V3 peptide were detected in animals inoculated with a combination of i.r. and i.d. routes Animals inoculated by combined routes had significantly higher titres of HIV-1-specific IgG and IgA in serum with enhanced neutralization activity Induction of high levels of IFN-γ and IL-2 mRNA in PBMC, splenocytes and intraepithelial lymphocytes up to 2 years after combination inoculation 	Kawahara <i>et al.</i> , 2002 ¹⁵⁴

HIV env V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus sequence)	rBCG-HIVEnv inoculated via oral route (80 mg of freeze-dried vaccine once a week for 4 consecutive weeks, providing a total dose of 320 mg)	guinea pigs	<ul style="list-style-type: none"> Env-V3 peptide-specific DTH responses were detected in immunized animals 1.5 years post inoculation Env-V3 peptide-specific proliferative responses detected in PBMC and intestinal intraepithelial lymphocytes indicating induction of functional T cells to HIV-1 Env in both systemic and mucosal compartments 	Kawahara <i>et al.</i> , 2002 ¹⁰⁶
SIVmac251 gag, nef and env (truncated) expressed individually in rBCG	Single i.d. inoculation (5×10^8 cfu) into 5 sites on the back, and rectal or oral boosting doses (6×10^{10} cfu) were administered intrarectally or intragastrically, respectively every 2 days for 6 days, (total dose of 18×10^{10} cfu). All the animals were challenged intrarectally with pathogenic SIVmac251	cynomolgus macaques (<i>M. fascicularis</i>)	<ul style="list-style-type: none"> After i.d. inoculation, monkeys exhibited CTL responses targeted against the three antigens and IFN-γ secretion was observed A rectal or oral boosting dose elicited anti-SIV IgAs in the rectum of vaccinated monkeys and increased IFN-γ secretion by circulating blood cells. No antibodies were detected and no CD4⁺ T-cell-mediated anti-SIV responses before challenge were observed No protection from SIVmac251 challenge 	Mederle <i>et al.</i> , 2003 ¹⁵⁵
HIV env V3 (encoding 19 amino acids of the Japanese HIV-1 V3 consensus) sequence	One s.c. immunization of 10mg rBCG env V3 and challenge from week 24 with either low-dose SHIV-MN, high-dose SHIV-MN, or SHIV-98.6PD at different intervals.	rhesus macaques	<ul style="list-style-type: none"> Significant levels of NAb for the 24 weeks tested that were predominantly HIV-1 type specific- neutralized primary HIV-1 isolates <i>in vitro</i> Neutralization was not observed against HIV-1SF33/X4 or primary HIV-1 R5 isolates Viral load in the vaccinated macaques was significantly reduced following low-dose challenge with SHIV-MN, and early plasma viremia was decreased after high-dose SHIV-MN challenge. Replication of pathogenic SHIV-89.6PD was not affected 	Someya <i>et al.</i> , 2005 ¹²⁷
SIVmac239 full length gag in rBCG-Tokyo downstream of hsp60 promoter	Animals were vaccinated with rBCG via i.d. by single inoculation (10 mg) and a replication-deficient vaccinia virus strain (DIs) expressing SIVmac239 <i>gag-pol</i> (rDIs-SIVgag) i.v. (10^6 pfu) in heterologous prime-boost or reverse combination. Control groups received	cynomolgus macaques (<i>Macaca fascicularis</i>)	<ul style="list-style-type: none"> rBCG-SIVgag/rDIs-SIVgag regimen induced high levels of SIV-specific IFN-γ spot-forming cells which afforded effective protective immunity against mucosal challenge with SHIV KS661c Other vaccination regimens including the opposite combination or the single-modality 	Ami <i>et al.</i> , 2005 ¹⁵⁶

	combinations containing rBCG with empty plasmid or DIs encoding β -galactosidase. All animals were challenged via intra-rectal inoculation with pathogenic 2000 TCID ₅₀ of SHIV KS661c, a derivative of SHIV89.6		combinations did not effectively protect against viral challenge	
HIV-1 clade A consensus gag (HIVA; containing a string of CD8⁺ T-cell epitopes) fused to <i>M.tb</i> 19-kDa signal sequence and expressed in a lysine auxotrophic strain of BCG	rBCG vaccination via i.p. (10 ⁶ cfu). Animals boosted with recombinant rMVA expressing HIVA (10 ⁶ pfu)	BALB/c Mice	<ul style="list-style-type: none"> rBCG prime-vaccination enhanced HIV-1 specific CD8⁺ T-cell responses induced by rMVA boost immunization Heterologous rBCG/rMVA regimen enhanced the vigour and quality of CD8⁺ T-cell response including that to a subdominant epitope This enhancement was directly related to dose, with higher doses effecting greater enhancement A DNA prime-rBCG boost regimen afforded protection against a surrogate virus challenge 	Im <i>et al.</i> , 2007 ¹⁵⁷
HIV-1 group M consensus envelope (CON6) either as a surface, intracellular, or secreted protein	rBCG vaccination via i.p. (either 10 ⁸ , 10 ⁷ , or 10 ⁶ cfu). Animals boosted with recombinant CON6 gp140 protein formulated in RiBi adjuvant via i.p. (50 μ g) Mice immunised with rAd5 and rVV in prime/boost combination served as positive controls	BALB/c Mice	<ul style="list-style-type: none"> rBCG-induced T-cell responses to HIV-1 envelope in spleen were lower than those induced by rAd/rVV prime/ boost rBCG induced comparable responses to rAd-rVV immunization in the female reproductive tract and lungs T-cell responses induced by rBCG were primarily CD4⁺, although rBCG alone did not induce anti-HIV-1 antibody However, rBCG could prime for a protein boost by HIV-1 envelope protein. Thus, rBCG can serve as a vector for induction of anti-HIV-1 consensus Env cellular responses at mucosal sites 	Yu <i>et al.</i> , 2007 ¹¹¹
Full-length SIV Gag protein under the control of <i>hsp60</i> promoter (rBCG-SIVGag) 0.5ng/mg	rBCG-SIVGag inoculated i.d by single inoculation of 0.1 mg or oral route (80 mg once a week for 2 consecutive weeks, providing a total dose of 160 mg)	guinea pigs	<ul style="list-style-type: none"> IgG2 levels greater than IgG1 IFN-γ mRNA expression detected in PBMC for both i.d. and oral vaccine groups i.d. rBCG induced DTH responses to PPD and SIV Gag p27 protein which were maintained for up to 50 weeks 	Kawahara <i>et al.</i> , 2006 and 2008 ^{112,158}

Full-length HIV-1 subtype C gag under the control of <i>mtrA</i> or <i>katG</i> promoters and 19-kDa localisation signal sequence	rBCG vaccination via i.d. (10^8 cfu) four times. All animals boosted via i.m. with HIV-1 subtype C Pr55 ^{gag} virus-like particles (Gag VLPs; 11 μ g) 1 year after last rBCG vaccination	chacma baboons (<i>Papio ursinus</i>)	<ul style="list-style-type: none"> ▪ Oral rBCG induced a long-lasting DTH response to the SIV Gag p27 protein, but not to PPD ▪ Induction of BCG vector-specific T-cell immune responses and recall responses after subsequent rBCG vaccinations ▪ Development of low but detectable T-cell responses to HIV-1 Gag after rBCG vaccination and which were efficiently boosted by Gag VLPs ▪ Generation of IFN-γ-producing CD3⁺ CD8⁺ T cells after rBCG/Gag VLPs prime/boost vaccination ▪ PBMCs from immunised baboons targeted peptides with documented HIV-1 Gag epitopes 	Chege <i>et al.</i> , 2009 ¹³²
Full-length SIVmac239 gag and pol and a modified env alpha antigen promoter and 19-kDa localisation signal sequence	rBCG vaccination via i.d. (10^6 to 10^9 cfu) or i.v. (10^9 , 10^8 , 10^7 , 10^6 cfu) twice, 23 weeks apart. All animals boosted via i.m. with rAd5 (10^{10} viral particles) expressing SIV Gag/Pol and Env once 20 weeks after last rBCG vaccination	<i>mamu-A*01</i> ⁺ rhesus macaques	<ul style="list-style-type: none"> ▪ Induction of BCG vector-specific T-cell immune responses with second rBCG vaccination showing a robust recall response ▪ Development of low but detectable T-cell responses to SIV Gag and Pol but not Env after rBCG vaccination. ▪ Generation of p11C tetramer-binding and highly polyfunctional CD8⁺T cells after rBCG/rAd5 prime/boost vaccination 	Cayabyab <i>et al.</i> , 2009 ¹⁵⁹
Whole HIV-1 CRF01_AE gag gene	Mice inoculated a single injection of 0.1 mg of the rBCG/HIV-1gagE vaccine via s.c. and i.d. Prime boost: prime with rBCG/HIV-1gagE 2×10^6 cells via s.c. and a boost rDIs/HIV-1gagE 1×10^6 pfu via i.v. or i.d.	BALB/c mice	<ul style="list-style-type: none"> • One month later higher CTL against various positions of the Gag protein by s.c. compared to that by i.d. • prime boost, increased CTL responses induced as noted by specific cell lysis • both routes sustained similar CTL levels seven months post immunization 	Promkhaykaew <i>et al.</i> , 2009 ¹⁴⁷

HIVA , an HIV-1 clade A-derived immunogen under the control of Ag85B promoter and linked to the 19 kDa signal peptide	Animals were primed with BCG.HIVA ⁴⁰¹ at 10 ⁶ cfu i.p. and boosted with MVA.HIVA i.m at 10 ⁶ pfu or OAdV.HIVA i.m at 10 ⁷ infection units (IU) after 12 weeks	BALB/c mice rhesus macaques	<ul style="list-style-type: none"> • BCG.HIVA⁴⁰¹ alone induced undetectable and weak HIV-1-specific CD8 T-cell response in mice and macaques, respectively • In a prime boost regimen robust and broad HIV-1-specific T-cell were induced • Multiple antigen-specific T-cell clonotypes were recruited into memory • Induction of persistent potent T-cell responses specific for PPD antigen 	Rosario <i>et al.</i> , 2010 ¹³¹
HIVA, an HIV-1 clade A-derived immunogen under the control of Ag85B promoter and linked to the 19 kDa signal peptide	Animals inoculated with 1x10 ⁷ cfu BCG.HIVA ⁴⁰¹ via i.d. and boosted with two i.m. injections of 5x10 ⁷ pfu MVA.HIVA after 11 and 14 weeks	neonate Indian rhesus macaques	<ul style="list-style-type: none"> • PPD response peaked at 4 weeks and declined thereafter • BCG.HIVA⁴⁰¹ alone elicited a strong BCG-specific response • In a prime boost regimen, BCG-specific IFN-γ-secreting lymphocytes were induced, however declined 23 weeks later • HIV-specific T-cell responses were detected 1 week after the first boost, but were very weak 	Rosario <i>et al.</i> , 2010 ¹⁶⁰
Trimeric HIV-1 V3 loop (mV3) under the control of <i>hsp60</i> promoter	Mice inoculated via i.p. 1x10 ⁷ cells/mouse and guinea pigs inoculated via i.p. 1x10 ⁸ cells/animal and boosted 4 weeks later with the same cells via the same route	BALB/c mice guinea pigs	<ul style="list-style-type: none"> • Induction of V3-specific antibodies, IgG2a was prevalent in the V3-specific antiserum • IgG1 and IgG3 were very low • DTH response was induced in guinea pigs • V3-specific T-cells in mice spleens, which were retained 5 months post-vaccination. 	Kim <i>et al.</i> , 2011 ¹³⁴

Abbreviations: Cytotoxic T lymphocyte (CTL); Delayed-type hypersensitivity (DTH); heat shock protein (*hsp*); intradermal (i.d.); intranasal (i.n.); intrarectal (i.r.); intravenous (i.v.); purified protein derivative (PPD); severe combined immunodeficiency (SCID); simian immunodeficiency virus (SIV)-HIV chimeric simian-human immunodeficiency virus (SHIV); simian-human immunodeficiency virus (SHIV-MN); highly pathogenic SHIV strain (SHIV-89.6PD); subcutaneous (s.c.).

1.2.3. FACTORS INFLUENCING THE IMMUNE RESPONSES TO RECOMBINANT BCG

There are a few factors that should be considered in developing rBCG vaccines, such as the immunization route, localization of the heterologous antigen and the dosage of BCG as these determine the type of rBCG immune response elicited post vaccination ^{148, 161, 162}. These factors are described in detail in the following sections.

1.2.3.1. Immunization route

Precise comparisons of immune responses elicited by rBCG administered through different routes are difficult since doses administered vary. Both antigen-specific humoral and cellular immune responses have been induced concurrently by the rBCG vaccines administered via combined routes. Kawahara *et al.*, (2002)¹⁵⁴ immunized guinea pigs with rBCG expressing HIV Env V3 via either a single (Intradermal (i.d.), intranasal (i.n.) and intrarectal (i.r.)) or combination (i.r./i.d. and i.r./ subcutaneous (s.c.)) routes. They observed humoral and cellular immune responses against both HIV-1 and tuberculin in systemic and mucosal compartments with all immunization routes, however, the animals inoculated with combined routes displayed significantly higher levels of immune responses compared to the animals inoculated via a single route.

Kawahara *et al.*, conducted two studies in 2006 and 2008^{112, 158}, in which they immunized guinea pigs with rBCG-SIVgag via i.d. and oral routes (Table 1.1). They observed long-lasting cellular and humoral immunity against both viral and bacterial antigens with both vaccination routes, which were maintained for up to 50 weeks. However, different kinetics of Gag-specific INF- γ responses were observed between the two immunization groups, 1 year post-immunization stronger responses were observed in the i.d. immunization group, whereas 3 years post-immunization the levels between the two groups became identical.

Studies have reported that mucosal compartments are the major sites of infection entry for viruses such as HIV and SIV ^{115, 163-165}. Furthermore it has been demonstrated that the gut-associated lymphoid tissue (GALT) is an important site for the interaction between the host and the pathogen during HIV infection, CD4⁺ T-cell depletion during the acute stage of infection and HIV replication during both the acute and the chronic stages of infection ^{115, 163-166}. Consequently

generating a vaccine that would be administered via the mucosal route could provide the first line of defence against the invasion of infection by inducing antigen-specific immunity at this primary route of transmission.

Indeed, studies in different animal models have shown that mucosal vaccination of rBCG is effective as it has been able to elicit strong long term antigen-specific humoral and cellular immunity^{111, 127, 133, 135}. For instance, after immunizing mice nasally with rBCG expressing HIV *env* V3, Hiroi *et al.*, (2001)¹³⁵ observed induction of high titres of Env V3-specific IgG antibodies, which were able to neutralize clinical HIV-1 isolates *in vitro*. Similarly in rhesus macaques Someya *et al.*, (2005)¹²⁷ reported that primary HIV-1 isolates were neutralized by high titres of HIV-1 type specific NAb that were elicited following immunization with rBCG expressing HIV *env* V3. With the aim to raise both of these immune responses against SIV, Lagranderie *et al.*, (1998)¹¹⁰ immunized BALB/c mice via different mucosal routes such as oral, aerogenic, nasal and rectal with a cocktail of three different rBCG strains each expressing large fragments of SIV_{mac}251 Nef, Env and Gag proteins. Although the vaccines induced local IgA, systemic IgG and CTLs against the three SIV antigens with all routes of immunization, they still observed some discrepancies in the responses. They concluded that the local and systemic immune responses induced with the rectal and oral routes were the most appropriate routes.

Considering the above studies, due to its innate adjuvant properties and the ability to induce CTLs and antibodies against HIV/SIV infections, BCG could be an ideal vaccine candidate for generating mucosal immunity.

1.2.3.2. Localization of the heterologous antigen and the impact on immunogenicity

A number of studies have highlighted the importance of heterologous antigen display in influencing the type of protective cell-mediated immunity elicited by BCG^{97, 99, 111, 167}. Generally the mycobacteria-derived antigens are processed and presented by the macrophages. Hess *et al.*, (2000)¹⁶⁸ reported that antigens delivered into the phagolysosome are usually processed via the major histocompatibility complex (MHC) class II pathway and thus induce predominantly CD4⁺ T cells. Consequently, in BCG-immunized animals the CD8⁺ T-cell responses induced are weak

¹¹¹.

Yu *et al.*, (2007)¹¹¹ hypothesized that the weak induction of CD8⁺ T-cell responses following BCG vaccination could be enhanced by processing the antigens expressed via the MHC class I pathway. It has been demonstrated in several studies that rBCG engineered to express heterologous antigens as membrane associated lipoproteins or as secreted proteins by fusing them to the secretion signal sequences induce better immune responses than the antigens localized in the BCG cytoplasm as these antigen display strategies ensure that the antigens are processed via the MHC class I pathway^{97, 99, 115, 157, 169}.

The *M. tuberculosis* 19 kDa lipoprotein signal is normally used to direct export and link the heterologous antigens to the BCG cell membrane⁹⁹. This lipoprotein is a cell wall or membrane-anchored protein and is highly immunogenic *in vivo*⁸⁰. Stover *et al.*, (1993)⁹⁹ immunized mice with an rBCG expressing the *Borrelia burgdorferi* outer surface protein A (OspA) antigen as a membrane associated lipoprotein. Their vaccine induced protective antibody responses that were 100 - 1000-fold higher than those elicited by rBCG expressing OspA in the cytoplasm or as a secreted fusion. Similarly Dennehy *et al.*, (2007)¹⁶⁷ showed that linking the heterologous antigen, VP6 protein to the 19 kDa lipoprotein signal sequence that transported it to the membrane was important for induction of protective immunity against rotavirus in mice.

The alpha (α) antigen from BCG or *M. kansasii* is usually used to export heterologous antigens¹¹³. Several studies have shown that this antigen exposure increases immune responses elicited^{97, 111}. Honda *et al.*, (1995)¹³³ inoculated guinea pigs and mice with rBCG secreting the HIV-1 V3 epitope (Table 1.1) via s.c. route. In guinea pigs following immunization and systemic passive transfer of the spleen cells they observed delayed-type hypersensitivity (DTH) responses to Env V3 and purified protein derivative (PPD) and HIV-specific antibody responses that were able to neutralize primary HIV-1 isolates. In mice CTLs were induced. Additionally the guinea pigs serum IgG effectively blocked HIV infection in severe combined immunodeficiency (SCID)/Hu mice.

Yu *et al.*, (2007)¹¹¹ immunized BALB/c mice with rBCG HIV-1 Env (Table 1.1). This antigen was expressed as an immunogen in different locations in BCG, i.e. either as a surface, intracellular or secreted protein. The secreted antigen induced the highest Env-specific T-cell IFN- γ responses. Using a similar system Grode *et al.*, (2002)⁹⁷ generated an rBCG expressing the immunodominant antigen p60 of *Listeria monocytogenes* in different bacterial compartments and

examined the cellular immunity in BALB/c mice. The anti-listerial protection evoked by the membrane-linked p60 lipoprotein and the secreted p60 were nearly equal and protected the mice from listeriosis, whereas cytosolic p60 failed to protect mice from listeriosis. In a subsequent study Grode *et al.*, (2005)¹⁷⁰ constructed an rBCG vaccine that secreted the membrane-perforating listeriolysin (Hly) of *L. Monocytogenes* and was deficient in urease C production (Δ ureChly⁺rBCG). Δ ureChly⁺rBCG showed improved protection against aerosol infection with *M. tuberculosis* compared to the parental BCG strain and improved antigen translocation into the cytoplasm.

Based on the above studies it is clear that expressing the antigen as a membrane-anchored protein or secreting it in BCG improves immunogenicity.

1.2.3.3. The dose of BCG

Most rBCG vaccine immunogenicity studies have implicated the dosage of BCG as a major factor influencing the T helper type 1 (Th1)/ T helper type 2 (Th2) nature of immune response elicited^{161, 169}. High doses of BCG deliver large amounts of foreign antigen, which favour higher humoral responses and lower cellular responses, and vice versa^{59, 162}. This is due to the fact that higher levels of antigen favour humoral immunity as the maturation of B lymphocytes is dependent on the constant presence of the antigen¹⁶².

In a murine model Langermann *et al.*, (1994)¹⁶⁹ demonstrated that the antibody response is dependent on the dose of BCG administered. Following immunization of the animals with rBCG expressing an outer-surface protein of *Borrelia burgdorferi*, OspA linked to the 19 kDa antigen, they observed that the lowest dose of the vaccine did not induce humoral responses, whereas the highest dose yielded high antibody titres. Power *et al.*, (1998)¹⁶¹ studied the effects of different doses of BCG on the type of immunity elicited in BALB/c mice. They reported that low doses elicited a Th1 response, whereas higher doses favoured a mixture of Th1/Th2 responses and a Th2-mediated humoral response. Additionally, In BALB/c mice Michelon *et al.*, (2006)¹⁶² showed that rBCG expressing the MSP1a antigen of *Anaplasma marginate* with an *in vivo* up-regulated vaccine vector induced significant seroconversion to MSP1a that was 26x above the pre-immunization levels and weak MSP1a specific Th1 responses, whereas a vaccine vector that was not up-regulated induced humoral immunity to MSP1a that was 6x above the pre-immunization levels, and markedly stronger cellular responses.

1.2.4. COMPLICATIONS IMPEDING THE EXPRESSION OF HETEROLOGOUS ANTIGENS IN RECOMBINANT BCG

In spite of its frequent use as a heterologous delivery system, there have been reports of complications associated with rBCG. In addition to causing disseminated BCG disease in immunocompromised individuals (mentioned in Section 1.1.2.2), *M. bovis* BCG grows extremely slowly *in vitro*, cultures have ~ 22 hr doubling time and colonies take up to ~ 4 weeks to develop on solid medium^{103, 171}. In liquid medium the strain aggregates, which hinders quantification of the cultures and minimizes the quality of the vaccine cultures¹⁷².

Low heterologous antigen expression and vector genetic instability are the two major issues accountable for reduction of the potency of the rBCG expressing viral antigens and prohibition of its commercial production^{95, 106, 125, 152}. These two issues are circuitously linked and can be attributed to a number of factors, which vary from the expression mechanism of the heterologous antigen to the codon compatibility between antigens and the host^{95, 103, 107, 173}. Low antigen expression and vector instability often result in induction of weak or no immune responses following vaccination.

1.2.4.1. Low heterologous antigen expression

Generally in recombinant bacteria, the introduction of foreign antigens require the resources (in the form of energy) of the host cells for maintenance and expression. As a result the metabolism of the host cells is disturbed¹⁷³⁻¹⁷⁶. When the heterologous antigen expression machinery uses up the host cell's resources it places a metabolic load on the host¹⁷³⁻¹⁷⁵. As a result the growth rate of the host cell is hindered^{95, 105}.

The factors that influence the metabolic load are the plasmid copy number and over-expression of foreign antigens^{176, 177}. Higher plasmid copy number leads to increased expression levels and therefore higher cellular energy utilisation. Over-expression of foreign antigens can lead to the formation of inclusion bodies and production of mis-folded proteins. This induces the expression of cellular proteases and chaperones in order to degrade these proteins^{173, 176, 178}. This degradation process increases toxicity within the cell and consumes cellular energy. The depletion of cellular energy becomes a metabolic burden^{103, 176}.

1.2.4.2. Genetic instability of the rBCG

Vector instability occurs during *in vitro* culturing and/or post immunization of mammalian subjects as a result of the metabolic load imposed by the heterologous antigen¹⁷⁶. It results in either of two processes. The first is structural instability, where there is an alteration or loss of the gene encoding the heterologous antigen due to mutation^{95, 103}. The second is plasmid inheritance instability where the entire plasmid is lost by the cells in the culture^{60, 106}. These processes result in a production shift in the culture, where the plasmid-less cells outgrow the plasmid-bearing cells^{173, 179}. This leads to low production of the heterologous antigens, which in turn lowers the immunogenicity of the vaccine and ultimately reduces protective efficacy¹⁷³. The rate at which genetic instability occurs is determined by the fitness level of the recombinants, which in turn is determined by the degree of the metabolic load¹⁷³. On condition that multiple passages are required for the large scale production of rBCG vaccines, vector instability makes the commercial production of the vaccine impractical¹⁷⁶.

1.2.5. STRATEGIES DESIGNED TO IMPROVE BCG AS A RECOMBINANT DELIVERY SYSTEM

1.2.5.1. Development of safer rBCG

As mentioned earlier, prior to the emergence of HIV/AIDS pandemic, BCG was considered safe, however now there are BCG safety concerns that have been raised since the reports of disseminated BCG disease in immunocompromised individuals have been published. Recent rBCG studies are aimed at developing rBCG vaccines that are safe for immunocompromised individuals, while maintaining the protective efficacy. Such vaccines could be used in areas with high HIV prevalence, where they could be administered for protection against TB to infants with unknown HIV status and individuals who are immune-compromised or HIV-positive.

Moreover due to reports of Mother-to-child transmission (MTCT) of HIV via breast-feeding^{157, 180}, these safe vaccines could be given to infants to prevent MTCT, where they would be administered at birth. Although studies have shown that maternal ARV therapy regimens and infant nevirapine can significantly reduce the risk of MTCT^{157, 180}, the high cost of these drugs prevents the constant supply in developing countries considering the drugs would have to be administered during the entire breastfeeding phase.

Auxotrophic strains of BCG have been recently constructed, whereby mutations are introduced into the strains to prevent them from synthesising essential growth compounds, e.g. a BCG mutant in which a gene encoding lysine is inactivated, is a lysine auxotroph. These auxotrophic strains are then unable to grow unless the lacked amino acid is added into the culture^{79, 157, 181}. Additionally auxotrophs have been shown to be safer than the parent BCG strains following administration to immune-deficient animal models^{32, 48, 55, 181}.

With the aim to develop an rBCG vaccine with improved safety and efficacy, Tullius *et al.*, (2008)⁵⁵ generated a rBCG ferrin mycobactin auxotroph expressing the highly immunoprotective *M. tuberculosis* 30 kDa major secretory protein rBCG(mtbB)30 and a rBCG pantothenate auxotroph expressing the same antigen (rBCG(panCD)30). They compared their vaccines to rBCG30 (a wild type rBCG strain expressing the same antigen) in terms of *in vitro* growth and immunogenicity in SCID mice and guinea pigs. The rBCG(mtbB)30 expressed the antigen at similar levels to the wild type rBCG30, however it induced better cellular immunity that conferred superior protection. The rBCG(panCD)30 on the other hand was comparable to rBCG30 in efficacy. Essentially both the auxotrophic vaccines were more attenuated than BCG and provided a safer alternative to the wild type BCG.

In attempt to develop a vaccine to protect infants from acquiring HIV-1 via breast-feeding, Im *et al.*, (2007)¹⁵⁷ generated a lysine auxotrophic of BCG (Table 1.1) and tested it in BALB/c mice. Their vaccine was very immunogenic and thus provided protection against aerosol challenge with *M. tuberculosis*. In a prime and heterologous boost regimen it elicited virus-specific responses that provided protection against surrogate virus challenge. They concluded that administration of this vaccine soon after birth may prime HIV-1 specific immune responses, which could be boosted with other vaccines expressing the same immunogen or by natural exposure to HIV-1 in the milk during breastfeeding, and thus reduce MTCT via breast milk.

Based on the above studies, it is clear that although auxotrophic strains are more attenuated, they still displayed immunogenicity comparable to that displayed by the parent BCG strains. Additionally, as mentioned in section 1.1.2 the tendency of BCG to sensitize for the tuberculin skin test is one of the limitations that prevent its extensive use. The results from a study conducted by Chambers *et al.*, (2000)³² reported that auxotrophs allow for the generation of vaccines that will not interfere with the tuberculin skin test. In their study they showed that

immunization of guinea pigs with a leucine auxotroph of BCG conferred considerable protection from *M. bovis* infection and against hematogenous spread of *M. tuberculosis* without developing a cutaneous DTH reaction to tuberculin. In a more recent study a safety profile of the auxotrophs was demonstrated, where Sampson *et al.*, (2011)⁴⁸ assessed the leucine and vitamin pantothenate auxotroph of *M. tuberculosis* in SIV-infected rhesus macaques, a good model for HIV-positive humans and observed no vaccine-associated adverse effects.

1.2.5.2. Improvement of rBCG stability and heterologous antigen expression

Evidently the potency of the rBCG vaccine is determined by the genetic stability of the vector and its ability to express sufficient heterologous antigen to induce a good protective immune response. Research is focusing on approaches designed to develop a potent rBCG vaccine. These include the use of single-copy integrative vectors, codon optimization, heterologous BCG primed-boost regimens and the use of inducible expression systems^{31, 64, 95}.

a. BCG shuttle vectors for the expression of heterologous antigens

Most episomal shuttle vectors were developed from a *Mycobacterium fortuitum* cryptic plasmid, pAL5000, in combination with an *E. coli* cloning vector, pIJ666 and a kanamycin resistance gene^{60, 110}. These vectors replicate at about five copies per mycobacterial cell^{90, 105, 182}. To address the problems of low antigen expression, episomal vectors have been used with constitutive promoters so that the heterologous antigens are constitutively expressed and are thus presented at levels sufficient to trigger immunity^{95, 157}.

In some studies episomal-based vaccines have displayed instability *in vitro*, this could be a result of metabolic load due to high heterologous antigen expression levels^{29, 176}. Furthermore, *in vivo* the unstable episomal vectors are unable to maintain stability in the absence of selection in cases where the antibiotic markers are used as selectable markers^{60, 91}. This is a disadvantage as the vaccinated subjects will have to be given antibiotics in order for the vaccine to remain stable^{21, 183}.

In order to overcome vaccine instability issues, integrative vectors were designed as alternative systems for expression of heterologous antigens^{95, 105, 184}. These vectors are derived from temperate mycobacteriophages such as L5 and Ms6^{60, 184}. They encode the integrase function that allows the phage *attP* to recombine with the bacterial homologous *attB* sites, which results

in the integration of the vectors into the mycobacterial genome^{60, 95, 184}. These vectors are better retained than episomal vectors¹⁶⁷. Additionally, integrative vectors are usually stable even in the absence of selection¹⁰⁵.

Since integrative vectors integrate into the mycobacterial genome as a single copy, heterologous antigen expression levels are reduced in comparison to episomal vectors^{167, 185}. However this expression reduction is counterbalanced by the *in vivo* stability of these vectors that allows the antigen synthesis to persist for long periods of time⁹⁵. Furthermore, optimum levels of heterologous antigen expression can be achieved by the use of integrative vectors in combination with strong promoters^{104, 105, 167}.

b. Selectable markers used in rBCG

The *E. coli*-mycobacterial shuttle vectors used in BCG vaccines for cloning and expression rely on the antibiotic resistance genes as selectable markers^{60, 89, 186}. Due to G-C rich thick and waxy cell walls of mycobacteria, it is not easy for many antibiotics to permeate through the walls. Thus antibiotics that can remain stable for prolonged incubations are required to facilitate the identification of recombinant mycobacteria^{60, 89, 186}. The problem is that these antibiotic resistance genes are unfavourable for *in vivo* based vaccines, given that to maintain stability selection antibiotics would have to be administered to the vaccinees.

To circumvent the above issue, expression systems that do not carry antibiotic resistance markers have been developed. Superinfection immunity to mycobacteriophages¹⁸⁷ and resistance to mercury¹⁸⁶ have been used as selectable markers in mycobacterial expression systems. However, these systems proved to be unsuitable for use in recombinant vaccine as they lack *in vivo* selective pressure^{60, 186, 187}. Consequently, the use of mycobacterial auxotrophs as selection markers has been investigated. The failure of auxotrophic strains to grow without the essential compounds provides for selection⁶⁰. The complementing genes are included on the plasmids in these auxotrophic strains to maintain the plasmids, which in turn improves stability. Borsuk *et al.*, (2007)⁶⁰ used unmarked homologous recombination to knock out the *leuD* gene of the BCG strain in order to generate a BCG auxotroph for the amino acid leucine. Expression of LeuD on a plasmid allowed complementation and also acted as a selectable marker. This system maintained the selective pressure, and as a result remained stable both *in vitro* and *in vivo*. Similarly Im *et al.*, (2007)¹⁵⁷ developed a rBCG vectored by a lysine auxotroph of BCG that

carried a plasmid with a *lysA*-complementing gene. They reported that complementation increased the plasmid stability *in vivo* and precluded mutation of the heterologous genes.

c. Codon optimization

Instability of *E. coli*-mycobacterium shuttle vectors may be attributed to the rare codons contained by the heterologous antigens¹⁸⁸. Expression of proteins with rare codons can also cause metabolic load, which leads to low antigen expression^{173, 189}. Codon optimization is a method that is developed as a solution to this issue. This method is applied to ensure that the codons of the heterologous antigen are compatible with those of the host expression system^{64, 107}. It has been applied to enhance foreign antigen transcription and translation in rBCG vector-based vaccines as well as immunogenicity in mammalian cells^{96, 107}.

Kanekiyo *et al.*, (2005)¹⁰⁷ observed a 40-fold increase in expression levels of HIV-1 p24 Gag gene in BCG after codon optimization. Additionally small doses of the codon optimized vaccine were able to induced HIV-1-specific cellular and antibody immune responses in mice. Applying this method Varaldo *et al.*, (2006)⁹⁶ were able to enhance the expression of a *Schistosoma mansoni* antigen, Sm14 by approximately 4-fold. However, immunity conferred by the optimized vaccine in an outbred Swiss mouse model against cercarial challenge did not improve, instead it was comparable to that conferred by the non-codon optimized vaccine.

As reported in the above studies codon optimization enhances the expression of the heterologous antigen, which implies that lower doses of the vaccine maybe sufficient to induce immunity. This could help alleviate the problem of low antigen expression that requires administration of high vaccine doses. However, there is no guarantee that the immunogenicity of the vaccine will be enhanced.

d. BCG prime with a heterologous boost

As reported in numerous TB studies (section 1.1.3.1), the heterologous BCG prime-boost regimen is more effective than a homologous regimen^{58, 78, 111}. Thus this regimen has been implemented in the development of rBCG vaccines. In some of these studies protective immunity against virus challenges has been conferred^{31, 156}. Ami *et al.*, (2005)¹⁵⁶ immunized cynomolgus macaques with a rBCG-SIVgag prime and a rDisSIVgag boost (Table 1.1) and elicited protective immunity against mucosal challenge with a pathogenic SHIV virus.

In other studies the use of the heterologous BCG primed-boost regimen has led to the induction of robust memory responses and production of enhanced antigen-specific CD4⁺ T cells and polyfunctional CD8⁺ T cells^{155, 159}. Chege *et al.*, (2009)¹³² reported that rBCG expressing the HIV-1 subtype C *gag* (Table 1.1) efficiently primed a HIV-1 subtype C (Pr55^{gag}) virus-like particles (Gag VLPs) boost in Chacma baboons as low T-cell responses to HIV-1 Gag were well boosted. Using the same approach in rhesus macaques and BALB/c mice Rosario *et al.*, (2010)¹³¹ demonstrated enhancement of HIV-specific T-cell responses following a prime with rBCG expressing an HIV-1 Clade A-derived immunogen (BCG.HIVA) and a boost with MVA-vectored or ovine adenovirus expressing the same antigen.

Overall, BCG vaccinations display good priming ability to the immune system seeing that robust anti-HIV-1 immune responses were generated in the above studies. Thus BCG vaccines administered via this regimen could potentially confer some protection against an HIV infection.

e. The use of inducible promoters

Another approach designed to solve the issue of constitutive expression that results in over-expression of heterologous antigens that increases metabolic load and reduces stability is the use of inducible systems^{173, 190}. Inducible systems are structures that allow for the control of gene expression in prokaryotes and eukaryotes, with the aid of inducible promoters. These systems will be explained extensively in the following section.

CHAPTER 2: A REVIEW ON REGULATION OF GENE EXPRESSION IN MYCOBACTERIA

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2.1. INTRODUCTION

Bacterial species exist in the environment that is very unstable and sometimes tends to be non-optimal for these species to grow properly. This instability is a result of nutrient variations and surrounding toxicity^{191,192}. Bacterial species have to adapt to these variations in order to survive. Their adaptation response is activated by regulated gene expression systems that enable them to modify their physiologies in order to respond rapidly and appropriately¹⁹²⁻¹⁹⁴. The regulation systems contain transcriptional regulators that respond to specific cellular and environmental signals and then trigger transcription, translation and gene expression mechanisms, which allow the modification processes to occur^{195,196}.

2.2. REGULATED GENE EXPRESSION SYSTEMS

The discovery of regulated gene expression systems was one of the greatest benefits to scientific research. These systems provide a way for manipulation and control of gene expression in prokaryotes as well as eukaryotes. This facilitates the genetic analysis of species and their biological processes, such as regulatory cascades and processes essential for growth^{191,193,197,198}.

The regulation systems can be utilised for facilitation of protein expression and purification, and identification and analysis of essential genes¹⁹⁹⁻²⁰¹. The key factor in these systems is the inducible promoters used to drive the expression of the target genes. These are normally strong promoters, which can be switched on or/and off under defined conditions^{197,202}.

2.2.1. REGULATED EXPRESSION SYSTEMS USED IN MYCOBACTERIA

The study of mycobacterial gene expression is complicated by the bacteria's long generation time that leads to low protein production. Due to extremely short generation time, *E. coli* and *Streptomyces lividans* are often used to host and express mycobacterial proteins²⁰³. However, given the high G-C content of mycobacterial DNA and post-translational modification of mycobacterial proteins, which tend to be rich in glycine, alanine, proline and arginine, translation and protein production are not always efficient in these strains^{171,199,204,205}. To facilitate protein production and purification in this species regulated gene expression systems were developed²⁰¹,

²⁰⁶. These systems include, the Acetamidase (Ami) ²⁰⁴, Transfer (Tra) ²⁰⁷, Pristinamycin-inducible ²⁰⁶ and Tetracycline-inducible (Tet) ^{197, 199} systems.

2.2.1.1. The Acetamidase system

The Acetamidase (Ami) system was discovered in *M. smegmatis* and was the first regulation system used in mycobacteria ^{204, 208}. The system uses the regulatory elements to control the expression of a highly inducible acetamidase, an enzyme that enables *M. smegmatis* to grow with aliphatic amides as the sole carbon source ^{190, 204}.

The Ami system consists of five genes that are driven by four promoters situated between them (Figure 2.1) ¹⁹⁰. The first is the *amiE* gene, which encodes acetamidase. *AmiE* is located downstream of and forms an operon with three other genes, *amiA*, *amiS* and *amiD* ^{204, 209}. *amiC* and *amiD* encode positive regulators, AmiC and AmiD, respectively. The expression of *amiC* is driven by the *P_c* promoter and that of *amiD* is driven by the *P2* promoter ¹⁹⁰. There is only one negative regulator, AmiA, which is encoded by *amiA* ^{208, 210}. Finally, *amiS* encodes a part of a putative ABC transporter system ¹⁹⁰.

The regulation of the Ami system is as complex as its structure ²⁰⁸. In the absence of acetamide, AmiA binds to the *P2* promoter, which prevents the RNA polymerase from binding thereby repressing the expression of *amiD*, *amiS* and *amiE* genes ²⁰⁴. However during the repressed state a basal level of transcription does occur from *P2* leakage. AmiA may also bind to *P_c* promoter, which causes a reduction in the expression of AmiC. In the presence of acetamide, AmiC interacts directly with the acetamide and AmiA, which causes a conformational change that prevents AmiA from binding to the *P2* promoter in so doing resulting in the expression of AmiD. As soon as it is expressed, AmiD activates the *P3* promoter, which drives the expression of acetamidase ^{190, 210}.

It has been reported that under induced conditions the acetamidase shows a high induction ratio and can account for up to 10% of the total protein production ^{190, 209, 210}. Furthermore, this enzyme has been used for overexpression of mycobacterial genes, antisense expression and for construction of conditional mutants of both fast and slow growing mycobacteria species ^{204, 209, 211, 212}.

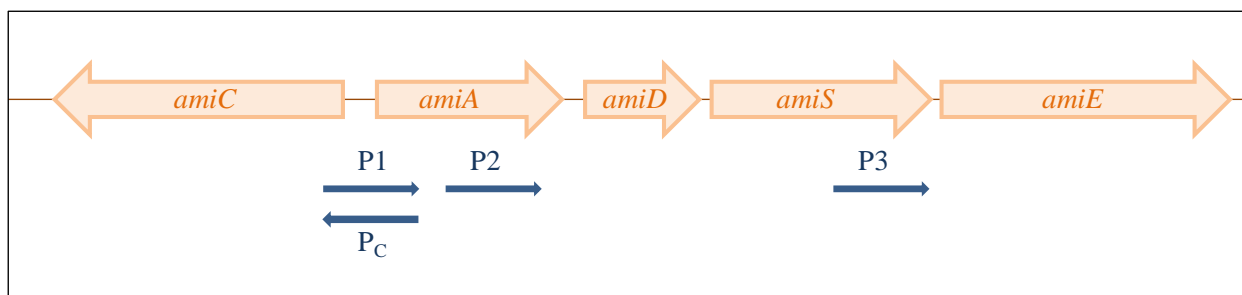


Figure 2.1: The gene organization of the acetamidase operon (modified from Parish *et al.*, 2001²¹⁰). This operon consists of *amiE* encoding acetamidase; an upstream *amiS* encoding a component of a putative transporter; *amiA* encoding negative regulator; and *amiC* and *amiD* encoding the positive regulators. These genes are controlled by the promoters located between them. In the absence of acetamide, AmiA binds to the *P2* promoter and prevents transcription of the *amiD*, *amiS* and *amiE* genes. In the presence of acetamide, AmiA dissociates from the *P2* promoter, which causes the transcription of downstream genes.

There are a few reasons that make the Ami system unsuitable for gene regulation studies, despite its highly inducible nature^{190, 213}. These include the large size of its operator that makes it difficult to manipulate, its complexity, and low stringency^{208, 214}. Additionally, the system is unsuitable for application to vaccine design seeing that under repressed conditions it allows basal expression of the target genes, which implies that genes under its control will always be expressed^{190, 210}.

2.2.1.2. The Transfer system

The Transfer (Tra) system was recently identified, it is essential for conjugative transfer of the 11-kb pSN22 plasmid in *Streptomyces nigrifaciens*²¹⁵. The system consists of a highly active inducible *Ptra* promoter and a repressor gene, *traR* encoding a temperature-sensitive TraR molecule²¹⁵. It is temperature sensitive and thus controlled by temperature changes. At temperatures $\leq 28^\circ\text{C}$, the TraR represses the initiation of transcription at the *Ptra* promoter. In contrast, at temperatures $>28^\circ\text{C}$, the TraR is inactivated, which leads to the induction of the *Ptra* promoter and transcription initiation²¹⁵.

The *Ptra* promoter has been used for high level heterologous expression of a *Streptomyces griseus* protease in *Streptomyces lividans*. Also, the Tra system has been employed in mycobacterial gene regulation. However, it is not ideal for controlling gene expression in mycobacteria given that it is activated at 28°C , a sub-optimal temperature for mycobacteria growth. Furthermore, the *Ptra* promoter does not allow very high protein production levels since it displays intermediate strength, a 15-fold reduction in activity compared to other mycobacterial

promoters, such as *hsp60*²⁰⁷. In the context of vaccine efficacy, the intermediate strength of the *Ptra* promoter would result in sub-optimal levels of antigen expression and consequently low immune responses.

2.2.1.3. Pristinamycin inducible gene regulation

The Pristinamycin (Pip) system was developed for gene expression control in eukaryotes and has been successfully used in plants and mammals^{216, 217}. Although it is not commonly used, the Pip system has been successfully adapted for use in both rapidly and slow growing strains of mycobacteria²⁰⁶.

The system consists of a very sensitive *Streptomyces coelicolor* Pip repressor that responds to the inducer and the *Streptomyces pristinaespiralis* *Pptr* promoter that drives the expression of the gene of interest^{206, 218, 219}. The clinically approved antibiotics that belong to the streptogramin group (pristinamycin, virginiamycin and Synercid) serve as inducers for the system. In the absence of the inducer, the Pip repressor binds to three operators overlapping the promoter region upstream of the *Pptr* promoter²¹⁷⁻²¹⁹. This binding results in the inhibition of transcription initiation, which is induced upon addition of the inducer as the repressor dissociates from the operators^{206, 217}.

Forti *et al.*, (2009)²⁰⁶ tested the system in the rapidly growing *M. smegmatis* and slow growing *M. tuberculosis* using the *lazZ* gene as a reporter, pristinamycin as an inducer and an integrative vector. They demonstrated that under induced conditions, the promoter activity increased up to 50-fold in *M. smegmatis* and up to 400-fold in *M. tuberculosis*. Complete repression of the promoter was observed in the absence of the inducer unlike in eukaryotes, where a low baseline expression was observed²¹⁷. Furthermore, by constructing conditional mutants of *M. tuberculosis* in the *fadD32* and *pknB* genes, Forti *et al.*, (2009)²⁰⁶ demonstrated that the system was highly effective as the strains did not grow in the absence of pristinamycin I indicating that the genes are essential for growth.

2.2.1.4. The Nitrile-inducible gene expression

This system originates from *Rhodococcus rhodochrous*, a saprophytic organism. This organism is used in the industrial production of acrylamide and nicotinamide^{205, 220}. The regulatory circuit of the system consists of the *nitA* gene encoding the nitrilase enzyme that detoxifies a broad

range of these compounds²²⁰. The expression of this gene is controlled by NitR²²¹. Seeing that NitR is homologous to AraC, a protein that serves as both positive and negative regulator of the arabinose operon, it is hypothesized that NitR is similarly regulated²⁰⁵. Nitrile and nitrile containing compounds act as inducers for this system, because in their presence, this organism produces large amounts of nitrilase enzyme encoded by the *nitA* gene. Under induced conditions, nitrilase can account for 35% of the total cellular protein²⁰⁵.

Since *Rhodococcus rhodochrous* is closely related to mycobacteria, Pandey *et al.*, (2009)²⁰⁵ hypothesized that the Nitrile system would be effective in mycobacteria. In their study they adapted the Nitrile system for use in mycobacteria to regulate the expression of genes in the fast growing *M. smegmatis* and the slow growing *M. tuberculosis*. Ehrt *et al.*, (2005)²²² reported that negatively-regulated promoters often produce basal levels of transcription. In order to avoid this low basal transcription Pandey *et al.*, (2009)²⁰⁵ designed a positive feedback system, in which the regulatory protein is induced simultaneously with the gene of interest. Their expression cassette contained the *nitR* gene, encoding the NitR under the control of *nitA* promoter and the gene of interest was driven by the second *nitA* promoter that was located in a separate operon. Their system achieved 100-fold induction in both mycobacterial strains using GFP as a reporter. Additionally, they also demonstrated that the system was not affected by the phases of growth and during growth in macrophages.

2.2.2. TETRACYCLINE INDUCIBLE (TET) SYSTEM

As mentioned above, most regulated gene expression systems have features that make them unattractive for the development of an inducible/ regulated recombinant vaccine. In this study we are using the tetracycline inducible (Tet) system for controlling gene expression in *M. smegmatis* and *M. bovis* BCG. Tet systems are derived from the Tn10-encoded Tetracyclines (Tc)-resistance determinant from *E. coli*²²²⁻²²⁵. Tc-resistance is said to be the most sensitive form of antibiotic resistance and is widely distributed in gram-negative bacteria^{198, 222, 225, 226}. To date Tet systems are the most widely used systems for conditional gene expression in prokaryotes and have been modified for use in eukaryotes^{225, 227}. These systems use Tetracyclines as inducers^{198, 199, 228, 229}.

Tetracyclines are a few of the most frequently used broad-spectrum of antibiotics, which kill cells by diffusing via the cell membrane^{230, 231}. In prokaryotic cells, these antibiotics inhibit

protein synthesis by preventing the attachment of the aminoacyl-tRNA to the ribosomal acceptor (A) site, which prevents the action of the 30S ribosome^{227, 230, 232}. In eukaryotic cells, tetracyclines inactivate the mitochondrial 30S ribosomes.

2.2.2.1. Tetracycline resistance

Tc-resistance occurs via two mechanisms, the first is ribosomal protection and the second is an efflux-based mechanism. In the former, Tet (O) a class of the ribosomal protection proteins (RPPs) dislodges Tc from the A site of 70S ribosome to restore polypeptide elongation and thus confers Tc-resistance to the bacteria^{227, 233}. The efflux-based mechanism confers resistance by transporting the drug out of the cell²²⁷. The efflux-based system is the most commonly used given that it has the most tightly regulated promoters and is used in this current study¹⁹⁸.

2.2.2.2. Efflux-based tetracycline resistance

The Tc-resistance determinant encoding active efflux is also referred to as the Tet system. The system consists of two genes, the resistance gene (*tetA*) and regulatory gene (*tetR*) oriented with divergent polarity^{226, 234, 235}. The two genes are separated by an 81 bp central regulatory region that consists of overlapping promoters, P_A, P_{R1} and P_{R2} from which they are transcribed²³⁶. Tet operators, *tetO*₁ and *tetO*₂ are also found in the regulatory regions (Figure 2.2)²²⁵.

tetA encodes a 36-kilodalton (kD) resistant protein, TetA, which is a membrane-spanning tetracycline/metal-proton (proton-[tc.Me]⁺) antiporter²³⁷. TetA is responsible for the export of Tc from the cell^{231, 237}. *tetR* encodes a 46-kD regulatory protein, TetR¹⁹². TetR is a dimeric DNA-binding repressor with two monomers, each consisting of 10 α -helices^{238, 239}. TetR contains N-terminal DNA-binding domains each has three helices (α 1 to α 3) that contain the α -helix-turn- α -helix (HTH)-motif, which mediates DNA binding to the operators^{226, 240}. TetR also contains a tetracycline binding region, which is located inside the C-terminal domain²⁴⁰.

2.2.2.3. Regulation of the Tet system

TetR has high affinity for the operators, and this binding to the operators tightly regulates the expression of both TetA and TetR^{234, 237, 240-242}. In the absence of Tc, TetR binds to the two operators with its DNA-binding domain, this binding prevents access of RNA polymerase to the promoter and thus inhibits transcription initiation resulting in the shutting down of the TetA and

TetR expression (Figure 2.2, panel A) ^{222, 225, 231}. The TetR-*tetO* interaction is said to be very strong, about $K_d \sim 10^{11} M^{-1}$, this is depicted by the complete repression in the absence of the drug ²⁴³. The presence of Tc reduces TetR-*tetO* interaction by nine orders of magnitude ^{243, 244}.

Tc crosses the cytoplasmic membrane by passive diffusion in a neutral form ^{240, 244, 245}. In the cytoplasm Tc forms complexes with the bivalent metal ions such as magnesium (Mg^{2+}), forming an active $[tc.Mg]^+$ complex (Figure 2.2, panel B). The $[tc.Mg]^+$ complex binds to the TetR-operator complex with high affinity of about $K_d \sim 10^9 M^{-1}$, which is much stronger than that for the ribosome of $K_d \sim 10^6 M^{-1}$ ^{222, 234, 246}. Binding of two molecules of $[tc.Mg]^+$ to a TetR dimer diminishes repressor affinity for *tetO* by causing a conformational change within the DNA-binding domain of the TetR, which then dissociates from the operators ^{222, 234, 247, 248}. This TetR dissociation from the operators results in the initiation of TetA antiporter expression ²²². The TetA antiporter exports Tc out of the system by exporting the $[tc.Mg]^+$ complexes. An export of each $[tc.Mg]^+$ complex out of the cell imports a proton simultaneously, which results in the reduction of Tc concentration within the cell (Figure 2.2, panel B). After all the Tc has been exported out of the cell, the remaining TetR binds to the *tetO*, which then shuts the expression of both Tet proteins ²²⁵.

TetR binds to the operator sites with different affinities. The affinity of TetR for *tetO*₂ is estimated to be 3 - 5 times higher than that for *tetO*₁ ²²⁶. Occupation of *tetO*₁ ensures a complete repression of both TetA and TetR ²²⁶. In contrast, occupation of *tetO*₂ ensures repression of the TetA expression, while allowing the P_R driven expression of TetR. This permits low level read-through of TetR, which provides a tightly regulated security circuit that prevents accidental expression of TetA (Figure 2.2) ^{225, 241, 244}. This regulation is important as it prevents long-term production of the TetA protein, which is said to be detrimental to the cell in the absence of Tc as it tends to be toxic at a high concentration ^{223, 249}. Essentially the expression of the TetA export protein is fine-tuned, in order to mediate Tc-resistance and at the same time avoid toxicity problems caused by its background expression ^{225, 250}.

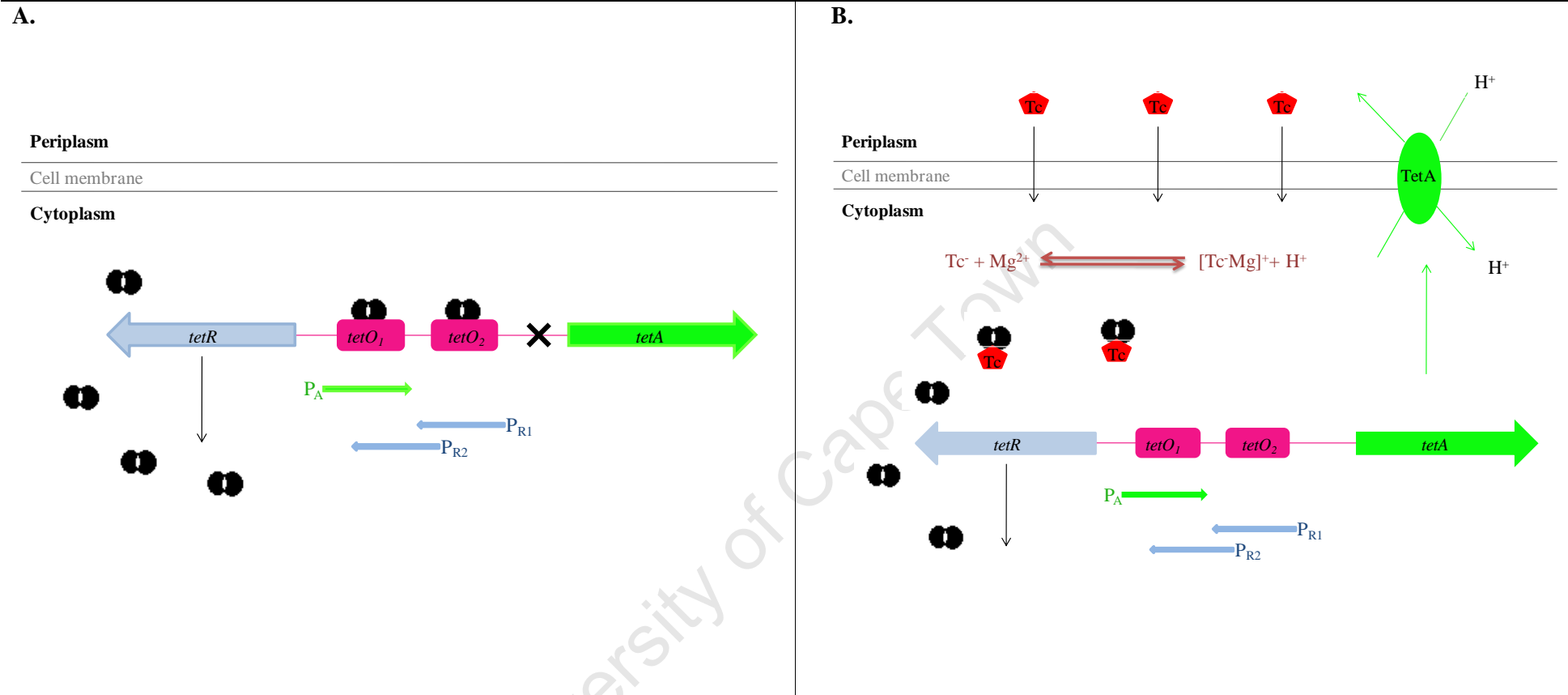


Figure 2.2: The genetic organization of the Tc-resistance determinant (modified from Hillen and Berens, 1994²²⁶). This system consists of divergently oriented *tetA* and *tetR* genes separated by a region of three overlapping promoters and two operator sequences. P_{R1} & P_{R2} promoters drive *tetR* expression and *tetA* expression is driven by P_A promoter. **A.** In the absence of Tc, TetR binds to the operators in the regulatory region, and this result in the repression of *tetA*. **B.** In the presence of Tc, *tetA* is expressed as the Tc binds to the TetR causing it to dissociate from operators.

2.2.2.4. Gene regulation by the Tet system

The Tet system is the most widely used tool for gene function studies. It has been successfully employed in both *in vivo* and *in vitro* studies^{202, 240}. The Tet system has a few advantages that make it the most popular tool for gene regulation, (i) The tetracyclines (the inducers of the system) are able to penetrate most cell walls and tissues, (ii) the system is highly sensitive, and thus low, nontoxic levels of the inducers are sufficient for regulation, (iii) the system allows the regulation and control of gene expression in liquid culture and during animal infections^{198, 199, 202, 226, 251}. Furthermore, the system has been adapted for gene regulation in gram-positive bacteria, acid-fast bacteria as well as eukaryotic systems such as human mammalian cells, transgenic animals and plants^{199, 250-253}.

2.2.2.5. Gene regulation by the Tet system in mycobacteria

Carroll *et al.*, (2005)¹⁹⁹ used the Tet system as a tool to determine the function of a *trpD* gene in *M. tuberculosis*. Firstly they showed that the system worked efficiently in *M. smegmatis* and *M. tuberculosis* using the GFP as a reporter and demonstrated a controllable on/off switch for fluorescence upon addition and removal of the inducer, anhydrotetracycline (ATc), a tetracycline derivative. They further generated a conditional auxotroph mutant of *M. tuberculosis*, where they used a Tc-inducible promoter to direct expression of the tryptophan biosynthetic enzyme, TrpD. Their results showed tight regulation of the *trpD* as the strain displayed auxotrophy by completely switching off the *trpD* only in the absence of tetracycline.

Ehrt *et al.*, (2005)²²² demonstrated ATc-dependency of the gene expression by the Tet system in *M. smegmatis* and *M. tuberculosis* in liquid culture. They further constructed a conditional gene knockout for a cell division essential gene, *ftsZ* in *M. smegmatis*. ATc concentrations 10- and 20-fold below the minimal inhibitory concentration for *M. smegmatis* and *M. tuberculosis*, respectively gave maximum gene expression. Additionally, they demonstrated gene regulation in *M. tuberculosis* within the macrophage phagosome.

Boldrin *et al.*, (2010)²⁰⁸ developed a repressible mycobacterial promoter system based on two different chromosomally encoded TetR and Pip repressors. They tested their system in *M. smegmatis* and *M. tuberculosis*, where they created conditional mutants of *ftsZ* and *fadD32*, respectively. The gene of interest was placed under the control of *Pptr*, which depends on the

pristinamycin I repressor. The Pip structural gene on the other hand was placed under the control of a TetR-dependent promoter. ATc was used as an inducer, such that its presence induced production of the Pip repressor leading to the repression of the *Pptr* promoter. Their results showed that both strains stopped growing in the presence of the inducer, which confirmed the essentiality of the knocked-out genes for growth. This demonstrated tight regulation by the system. All in all their system showed high stringency and versatility.

2.2.2.6. Limitations of the Tet system

Despite the efficiency of the system, there are some practical drawbacks that have been stated. Tetracyclines are nontoxic to cells at minimal levels, however in Tet systems the antibiotics serve as transcriptional inducers and thus have to be present continually. Vaccinees given vaccines harbouring the Tet systems would have to be given the tetracyclines to activate the expression of antigens, which is undesirable in humans and transgenic animals and for gene therapy. The continuous presence of the inducers makes the experiments sub-optimal^{225, 252}. Furthermore, according to Berens and Hillen, (2004)²⁵⁰ the constant presence of the tetracyclines leads to accumulation of resistance, depot-building in the organism and if present in gestation may result in reduction in transgene expression levels. An additional disadvantage is that in conditional knock-down systems, tetracyclines have to be removed from the bacterial culture in order to initiate gene silencing. The removal process is very slow and difficult as it requires procedures that may affect biological processes under investigation^{197, 198, 252}.

To overcome the above problems the reverse Tet mutants that require tetracyclines as co-repressors were developed^{197, 198}. A reverse Tet (revTet) system allows the expression of the target gene to occur in the absence of tetracyclines and permits gene silencing in its presence^{198, 250}.

2.2.3. REVERSE TETRACYCLINE (REV_{TET}R) SYSTEM

Scholz *et al.*, (2003)²⁴¹ demonstrated that mutations can be introduced in the *tetR* gene to change the inducer specificity of the TetR protein. This was done by random mutagenesis where three amino acids, E15A, L17G and L25V within the α -1 helix of the DNA-binding domain were mutated. These changes reverse the allosteric response of the binding of TetR to Tc, whereas

binding of Tc to the wtTetR causes the repressor to dissociate from the operators, binding of Tc to mutated TetR (revTetR) causes the repressor to bind to the operators^{198, 238}.

In 2004 Scholz and colleagues²⁵⁴ introduced different mutations in the *tetR* gene and generated more than 100 revTetR mutants. They tested the allosteric response of the revTetR mutants to the inducer. While most contained 2 - 5 amino acid mutations, some contained only 1 mutation and still showed a reverse allostery. Several reverse Tet mutants were characterised and analyzed by Berens and Hillen, (2004)²⁵⁰ in *E. coli* and *Bacillus subtilis*. One of the mutants, TetRr1.7 was selected, it contained the E15A, L17G and L25V mutations. The TetRr1.7 mutant showed the most efficient regulation in *E. coli*, switching off target gene expression completely upon the addition of ATc²⁵⁴.

The newly developed revTetR mutants were tested in gram positive bacteria by Kamionka *et al.*, (2005)²⁵⁵, where they wanted to expand the regulatory window of the Tet promoter in *B. subtilis*. Both wtTetR and the revTetR were used. In the presence of 0.4 μ M ATc, the highest induction obtained with the wild type system was 300-fold, and a 500-fold gene knockout was observed with the revTetR system.

2.2.3.1. Regulation of the RevTetR system

Once it is in the system Tc binds to the revTetR protein, which results in the formation of a Tc-revTetR complex. A conformational change occurs to the DNA binding domain of the revTetR protein, which enables the Tc-revTetR complex to bind to the operator region. The occupation of the operators leads to the shutting down of TetA expression. Upon removal of Tc, revTetR dissociates from the operators, thus allowing TetA expression^{222, 238, 255}.

2.2.3.2. Gene regulation by the RevTetR system in mycobacteria

The revTet system has been used repeatedly in mycobacteria to generate Tc-dependent conditional gene knockouts to study gene function. Gandotra *et al.*, (2007)²⁰⁰ used the TetRr1.7 mutant to generate a conditional knock-down mutant of the *M. tuberculosis* growth essential gene, *prcBA*. The gene was silenced *in vitro* by adding ATc. They were able to determine the function of the *prcBA* encoded core proteasome, as the silencing of the gene uncovered the requirement of the proteasome for virulence at a chronic phase of infection in mice. In their study Guo *et al.*, (2007)¹⁹⁷ used the TetRr1.7 to construct a conditional mycobacterial mutant of

an *M. smegmatis* growth essential gene, *secA*. The SecA1 protein was efficiently repressed in the presence of as low as 10 ng/ml of ATc, which demonstrated the sensitivity of the system. Additionally, a 90-fold decrease of the cell envelope associated porin, MspA was observed after the silencing of the *secA* gene. MspA was identified as the substrate of a secretion system. Based on their results, it is clear that gene silencing can be used for identification of substrates involved in biological processes.

It was previously reported that repression by the Tet system requires high levels of the revTetR protein, which occurs if the TetRr1.7 gene is expressed in a multicopy plasmid and transcribed by a strong mycobacterial promoter¹⁹⁸. In order to improve repression by the revTetR systems, Klotzsche *et al.*, (2009)¹⁹⁸ codon optimised the revTetRs by adapting the codon usage of the *tetR* gene to that of *M. tuberculosis*. This resulted in the formation of new revTetR mutants, which did not require a multicopy plasmid and a strong promoter for efficient expression. They tested the codon optimized mutants in *M. smegmatis* and *M. bovis* BCG. Upon the addition of ATc, the mutants mediated up to 50-fold repression in both strains. Additionally, complete silencing of the *M. smegmatis secA* gene by chromosomally integrated revTetR mutants was achieved.

2.2.4. THE USE OF THE TET SYSTEMS IN EUKARYOTES

2.2.4.1. The rTA (Tet-off) system

Due to its success in gene function and biological studies in prokaryotes, the Tet system has been modified for application in eukaryotes. These systems were introduced to eukaryotes to provide a way to regulate gene expression directly by changing experimental conditions^{256, 257}. Since regulation systems are operated by inducible promoters, they provide a good tool for *in vivo* gene function studies in transgenic animals^{256, 258}. In these studies, the system is useful for conditional inactivation and over-expression of the genes in a tissue-specific and time-dependent manner²⁵⁸.

Gossen and Bujard, (1992)²⁵⁹ first described a Tet system that was adapted for use in mammalian cells. They converted the TetR into a tetracycline controlled transcriptional activator (tTA) by fusing the herpes simplex virus (HSV) - VP16 activation domain and the TetR-tet operator mutant from *E. coli*^{252, 253, 260}. The tTA stimulates transcription from a minimal inducible human cytomegalovirus (P_{hCMV-1}) promoter combined with seven operators (*tetO*)²⁵⁹. Gene expression from this promoter is tightly controlled by the presence or absence of tetracycline and its

derivatives^{259, 261}. The original system was based on the presence of two plasmids, a constitutive plasmid expressing the tTA and a plasmid containing the target gene under the control of an inducible promoter containing the operators upstream of the TATA box^{259, 262}. The TetR moiety provides an inducer-dependent conditional DNA-binding domain that mediates the docking of the VP10 to a synthetic promoter²⁶². The binding of the tTA to the promoter and subsequent transcription of target gene is inhibited by the presence of Tc^{253, 259}.

Gossen and Bujard, (1992)²⁵⁹ were the first group to test the tTA system. They use the tTA to regulate the luciferase gene in mammalian HeLa cell lines. The results showed that the luciferase activity can be regulated up to 10⁵-fold by adding and removing Tc. In their study, a Tc derivative, doxycycline (Dox) was used as an inducer.

Paulus *et al.*, (1996)²⁶⁰ aimed to deliver as well as control the expression of heterologous genes in mammalian cells. They developed a retroviral vector that enclosed both response and regulatory units of the Tet system. They tested the vector in different mammalian cell types, by regulating the luciferase activity using Tc to control the system. Tc-dependent regulation was observed in all cell types transduced with the vector. The highest induction was observed in infected NIH 3T3 cells, where the levels in the absence of Tc were about 340-fold higher than the levels in the presence of the drug.

2.2.4.2. The rtTA (Tet-on) system

Four amino acid changes were made in the TetR moiety of the tTA to form the reverse tTA (rtTA) system, which changed the *tetO* binding²⁵². In the rtTA system the transactivator requires the presence of tetracyclines to bind to *tetO* in order to activate the expression of the target gene.

The rtTA has been shown to efficiently regulate the expression of exogenous genes in many eukaryotic systems, such as, transgenic mice and tissue culture etc.^{261, 262}. Although both rtTA and tTA systems have shown similar extents of transgenic expression, the induction kinetics of the tTA are slower given that the tetracyclines have to be removed completely to achieve the highest induction, which makes the rtTA a faster protein expresser²⁵⁸.

An improved Tc-dependent reverse transactivator, rtTA2^S-S2 was recently developed for transgenic animal studies^{258, 261}. The rtTA2^S-S2 has the same induction kinetics as the rtTA, but

has a 10-fold enhanced regulation, higher sensitivity to tetracyclines and no residual binding affinity to the operators in the absence of tetracyclines and thus lower background activity^{258, 263}.

In 2003 Gallagher and colleagues²⁵⁸ showed that the rtTA2^S-S2 system can be tissue-specific. They used the system to control protein synthesis in mice kidneys. They used the rTA^{LAP}-1 mouse line, which were transgenic mice that produce the rtTA under the control of the liver activator protein (LAP) promoter. They demonstrated the tightly controlled and reversible expression in the kidneys and livers by using the reporter lines with luciferase and the *LacZ* genes by adding and removing doxycycline (Dox). Dox was added in the drinking water. In the kidneys the transgene expression was observed in cortical proximal tubules. No luciferase and β -galactosidase activity were observed in mice that were not given Dox or after withdrawal of Dox. By exposing the rtTA producing mothers to Dox before mating, they showed that sufficient amounts of Dox cross the placental barrier during development of the foetus as the kidneys and livers of the newborns showed transgene activity.

2.2.5. SENSITIVITY OF THE T_{ET} SYSTEMS TO TETRACYCLINES

Due to their non-toxicity Tetracyclines have been clinically approved for administration to animals as well as humans^{208, 216}. In the current study ATc is used as a co-repressor. This Tc derivative is the most commonly used drug²⁵¹. ATc has numerous properties that make it a superior compound (Figure 2.3): (i) it has a high functional stability in cell culture, (ii) it is a more active and efficient inducer than all the Tc derivatives given that the binding constant of the TetR for [ATc·Mg⁺] is $K_d \sim 10^{11} \text{M}^{-1}$, which is higher than the one for [Tc·Mg⁺] of $K_d \sim 10^9 \text{M}^{-1}$, (iii) ATc has higher minimum inhibitory concentration (MIC) than Tc and (iv) ATc is the least toxic derivative of Tc^{208, 225, 243, 251}. Therefore ATc levels required to repress the expression of the transcription of the target gene are 10-fold below the levels required for protein inhibition^{249, 251}. Moreover, using HeLa cells Gossen and Bujard, (1993)²⁵¹ showed that concentration at which the prolonged ATc presence begins to affect the growth rate of the cells in culture is more than 1000-fold above the effective concentration.

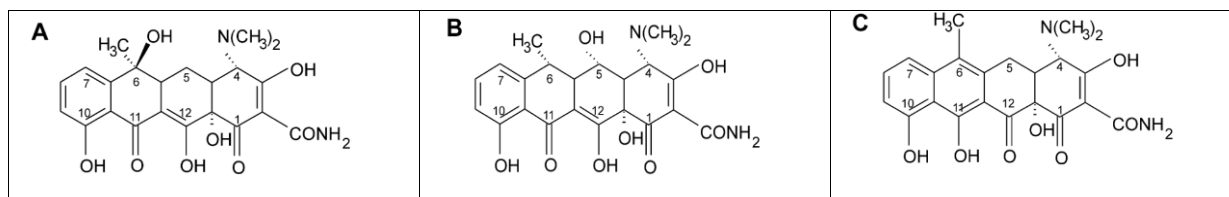


Figure 2.3: Structures of tetracyclines for gene regulation in prokaryotes and eukaryotes²²⁷. A. Tetracycline. B. Doxycycline, a derivative that is commonly used in transgenic animal studies. C. Anhydrotetracycline, a derivative that is commonly used in prokaryotic studies.

2.2.6. AIMS OF THE THESIS

Our research group is working on developing *M. bovis* BCG as a vaccine vector, specifically to express HIV-1 antigens. The successful development of a recombinant *M. bovis* BCG as an HIV vaccine (rBCG-HIV) is going to depend largely on overcoming instability associated with the expression of viral antigens. **The aim of this study is to utilise the tetracycline dependent gene regulation system to down-regulate recombinant antigen expression in BCG during the expansion of seed stocks and up-regulate expression prior to or post vaccination.**

This project will investigate whether the reverse tetracycline dependent regulatory system could be functionally developed for use in BCG using a multicopy episomal plasmid. The design is based on constitutively expressing the DNA binding regulatory protein revTetR, such that in the presence of the analogue anhydrotetracycline (ATc) in the media, the revTetR-ATc complex would bind *tet* operator sites placed within the *M. smegmatis* derived P_{smyc} promoter and result in shutdown of antigen expression during *in vitro* growth. In this way it could be tested if rBCG seed stocks can be expanded in the presence of ATc and if the vaccine remains stable. When tetracycline is removed the rBCG should express antigen at a higher level. The system will first be optimized using GFP as a model antigen, and then tested using HIV antigens. The most stable HIV antigens will be tested for immunogenicity in mice. **The application of this system in rBCG vaccine design is completely novel, and promises to significantly alleviate the problems associated with instability.**

CHAPTER 3: CONSTRUCTION AND ASSESSMENT OF THE EPISOMAL T_{ET}Rr1.12 DEPENDANT REGULATORY SYSTEMS

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3.1. INTRODUCTION

Ideally the rBCG vaccine should express the heterologous antigen at low levels during *in vitro* culturing in order to avoid over-expression of the antigen within the bacteria that would cause vector instability. Subsequently, the expression should be induced after vaccine administration to ensure that a sufficient amount of the antigen is produced in order to induce a strong protective immune response.

We aim to investigate a candidate rBCG HIV-1 subtype C vaccine that utilises the reverse (rev) TetR inducible system, derived from an *E.coli* Tn10-encoded Tetracycline (Tc)-resistance determinant²²² to regulate the expression of the recombinant HIV antigen. Our rBCG vaccine will down-regulate the HIV antigen expression during *in vitro* preparation of seed stocks and up-regulate it prior to and/or post vaccination.

As reviewed in Chapter 2, Scholz *et al.*, (2004)²⁵⁴ developed 100 reverse TetR repressor mutants and determined their efficiency by analysing their ATc-dependent expression using β -galactosidase (β -gal) as a reporter gene. The efficiency of the mutants was measured as an induction factor, which was given as the ratio of β -gal activity in the presence and absence of ATc. The most efficient mutants were the TetRr1.7 and TetRr1.12, which had induction factors of 102 and 90, respectively. The TetRr1.7 repressor is encoded by the *tetRr1.7* gene, which has the following amino acid mutations in the protein sequence, E to A at position 15; L to G at position 17; L to V at position 25 (Appendix D1) and the TetRr1.12 repressor is encoded by the *tetRr1.12* gene with amino acid mutations, V to G at position 20; G to R at position 21; I to N at position 22 in the protein sequence (Appendix D2).

In our laboratory the *tetRr1.7* and *tetRr1.12* mutants were codon optimized for regulation purposes in mycobacteria, and placed under the control of the strong *M. tuberculosis hsp60* promoter to generate the TetRr1.7 and TetRr1.12 expression cassettes, respectively (Dr Helen Stutz). The TetRr1.7 repressor has been utilised for gene silencing purposes in mycobacteria, where it has demonstrated excellent repression proficiency in the presence of the co-repressor, ATc^{197, 198, 200}. In 2009, Mayat²⁶⁴ constructed different shuttle plasmid designs of the episomal TetRr1.7 dependant regulatory system by inserting the TetRr1.7 cassette into the pNM3 and pNM4 vector backbones (Appendix D3.1) in different orientations as illustrated in Figure 3.1. Four designs of the episomal TetRr1.7 plasmids resulted (Appendix D3.2), these constitutively

express the TetRr1.7 protein from the *hsp60* promoter. The mechanism in which the TetRr1.7 dependent regulatory system regulates the expression of recombinant antigens is depicted in Figure 3.2. She explored the potential of the TetRr1.7 plasmid designs to control the expression of a reporter protein, a jellyfish *Aequorea Victoria* green fluorescent protein (GFP) within the model organism *M. smegmatis*, i.e. the repression of GFP expression in the presence of ATc and induction in its absence. In the pilot experiment a concentration of 100 ng/ml of ATc was used. The results confirmed the concept of the study by showing that the reporter GFP could be repressed by ATc by approximately 3-fold. The maximum repression of the systems occurred 8 hours after the addition of ATc.

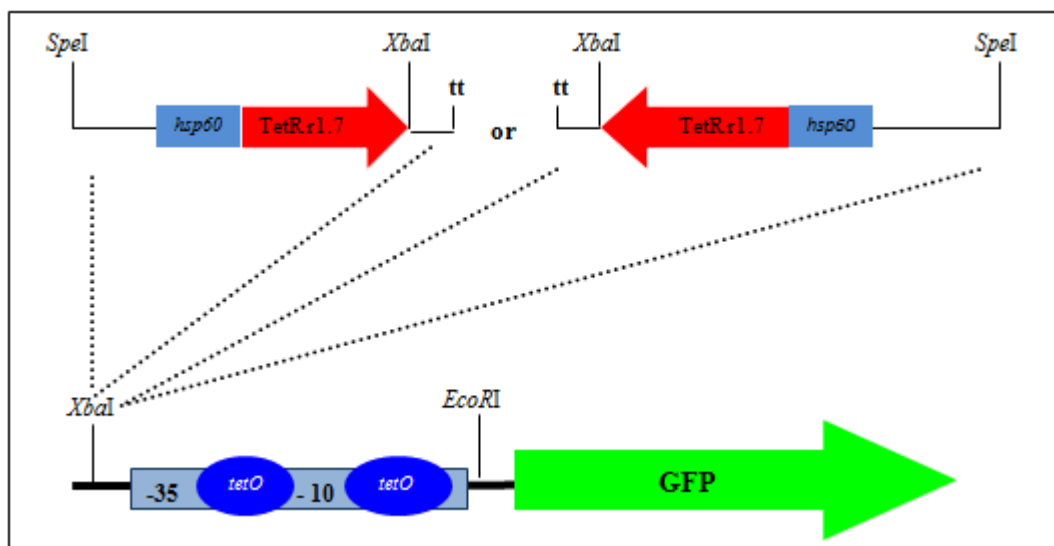


Figure 3.1: Strategy for the insertion of the TetRr1.7 cassette into selected pNM3 and pNM4 vector backbones (Modified from Mayat, (2009)²⁶³). The TetRr1.7 cassette, comprising the *hsp60* promoter, the *tetRr1.7* regulatory gene and the *hsp60* transcriptional terminator (tt) was cloned into the *XbaI* site of the vectors in either orientation.

Using different concentrations of ATc and monitoring the induction 12 hours after its removal, Mayat, (2009)²⁶⁴ demonstrated that the induction of GFP expression occurred in a concentration dependent manner. The cultures that contained lower concentrations of ATc achieved the highest induction, whereas cultures that contained higher concentrations of ATc did not produce as higher levels of GFP. *rM. smegmatis*[pNM3] and *rM. smegmatis*[pNM4] showed no evidence of GFP repression in the presence of ATc or induction after its removal, which was expected since these plasmids do not contain the TetRr1.7 cassette to allow regulation.

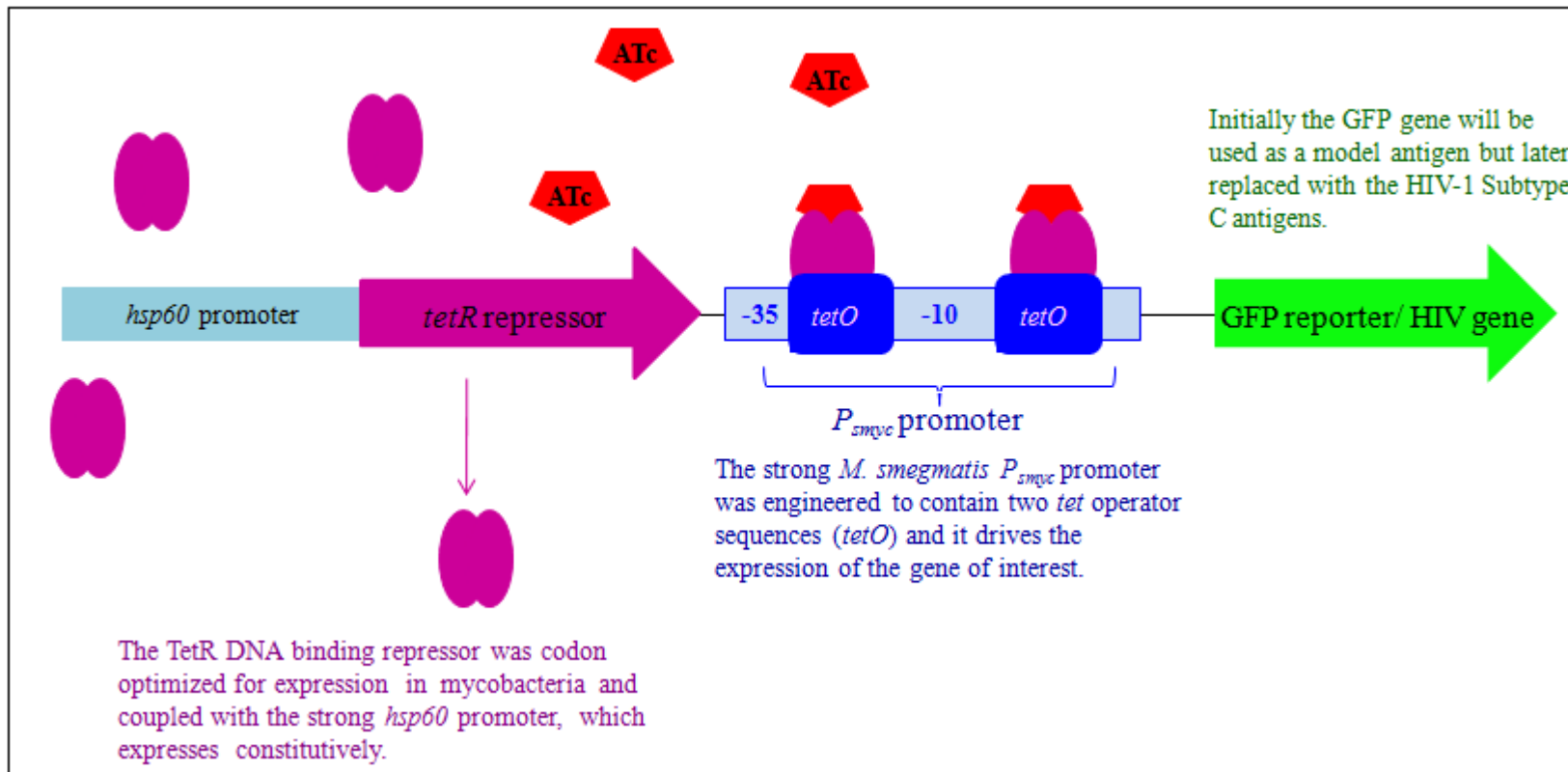


Figure 3.2: Diagram illustrating the design of the TetR dependant regulatory system (modified from poster presented by Dr Helen Stutz). In the presence of ATc, TetR-ATc forms and binds to the *tetO* operator sequences of the *P_{smyc}* promoter, which blocks the promoter activity, and thus the expression of the gene of interest is repressed. In the absence of ATc, the TetR regulator protein cannot bind to the *tetO* operator sequences, thus the expression of the gene of interest is induced.

3.1.1. STUDY OBJECTIVES

There is no published data on the TetRr1.12 mutant, thus it was selected for regulation of recombinant antigen expression in this study. The objective was to develop, evaluate and optimize the regulatory system using the TetRr1.12 repressor in the model organism *M. smegmatis*. Next would be to introduce and evaluate the system in the pantothenate auxotrophic strain *M. bovis* BCG mc² 6000 (*M. bovis* BCG $\Delta panCD$). In 2009, Mayat²⁶⁴ performed a 12 hour assay to monitor the induction of GFP expression after ATc removal and did not achieve complete induction, therefore in this study we aim to increase the assay period and monitor both GFP expression and genetic stability. The experimental plan is depicted graphically in Figure 3.3.

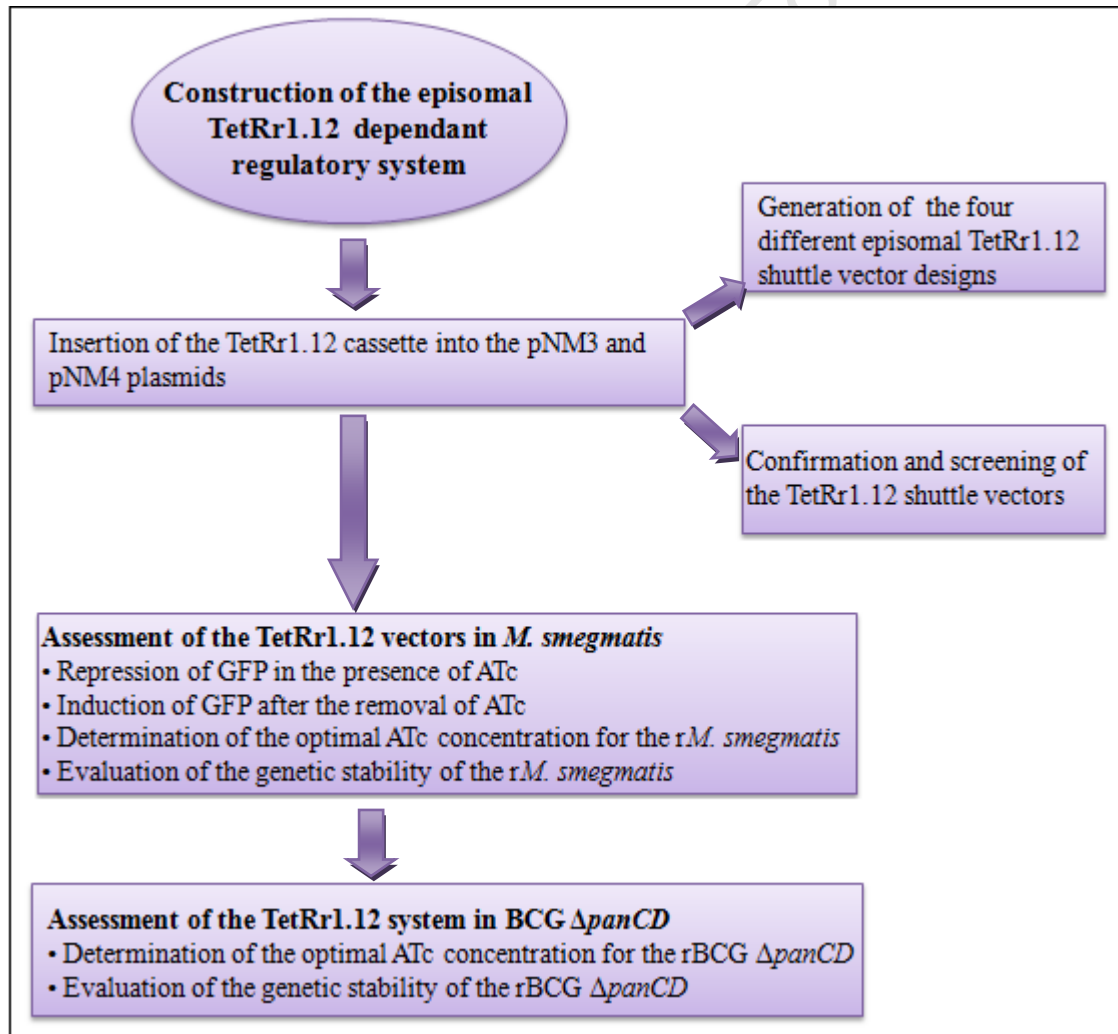


Figure 3.3: A schematic illustration of the project plan for this Chapter.

3.2. MATERIALS AND METHODS

3.2.1. GENERATION OF THE *E. coli*/MYCOBACTERIAL SHUTTLE PLASMIDS

3.2.1.1. Generation of the parental mycobacterial shuttle vectors

The *E. coli*-mycobacterial shuttle plasmid, pRC100 (Appendix E1) constructed by Dr Ros Chapman was used to construct the parental mycobacterial shuttle vectors, pNM3 and pNM4. The pRC100 vector contains a kanamycin resistance (Kan^R) gene, a mycobacterial origin of replication (*oriM*) derived from the pAL5000²⁶⁵, an *E. coli* origin of replication (*oriE*) and the *hsp60* promoter that drives the expression of GFP placed downstream.

The *hsp60* promoter in the pRC100 plasmid was replaced with a strong *M. smegmatis* *P_{smyc}* promoter (290 bp). The *P_{smyc}* promoter was derived from that reported by Ehrt *et al.*, (2005)²²² and engineered to contain *tetO* elements that facilitate the regulation of the expression of the gene of interest. Different combinations of operator sequences, *tetO*₁ and/or *tetO*₂ were designed flanking the -10 region of the *P_{smyc}* promoter as shown in the Appendix D3.1, which resulted in two parental mycobacterial shuttle vectors, pNM3 and pNM4 (Appendix E4 & 5).

3.2.1.2. Cloning of the TetRr1.12 expression cassette into the parental vectors

The mycobacterial shuttle vectors, pNM3 and pNM4 were isolated from *E. coli* using the large-scale plasmid isolation procedure (Appendix B1.2), and linearized with the restriction enzyme *Xba*I as described in Appendix B2. The TetRr1.12 expression cassette was synthesized and supplied by GeneArt® [Germany]. The cassette was excised from the GeneArt® [Germany] pGA1 vector backbone (Appendix E3) by a double digest with the flanking restriction enzymes *Xba*I + *Spe*I [Roche], which generated compatible ends for cloning into the parental plasmids, pNM3 and pNM4 digested with *Xba*I (as shown in Figure 3.4). All digested fragments were gel purified using QIAquick Gel Extraction Kit [Qiagen] according to manufacturer's instructions and quantified on 1% agarose slide gel by comparison to known concentrations of λDNA. The cassette was cloned into pNM3 and pNM4 using the ligation procedure described in (Appendix B4). It was inserted immediately upstream of the *P_{smyc}* promoter in two different orientations with respect to the *gfp* gene, and this resulted in the formation of four different episomal TetRr1.12 shuttle vector designs (Figure 3.4).

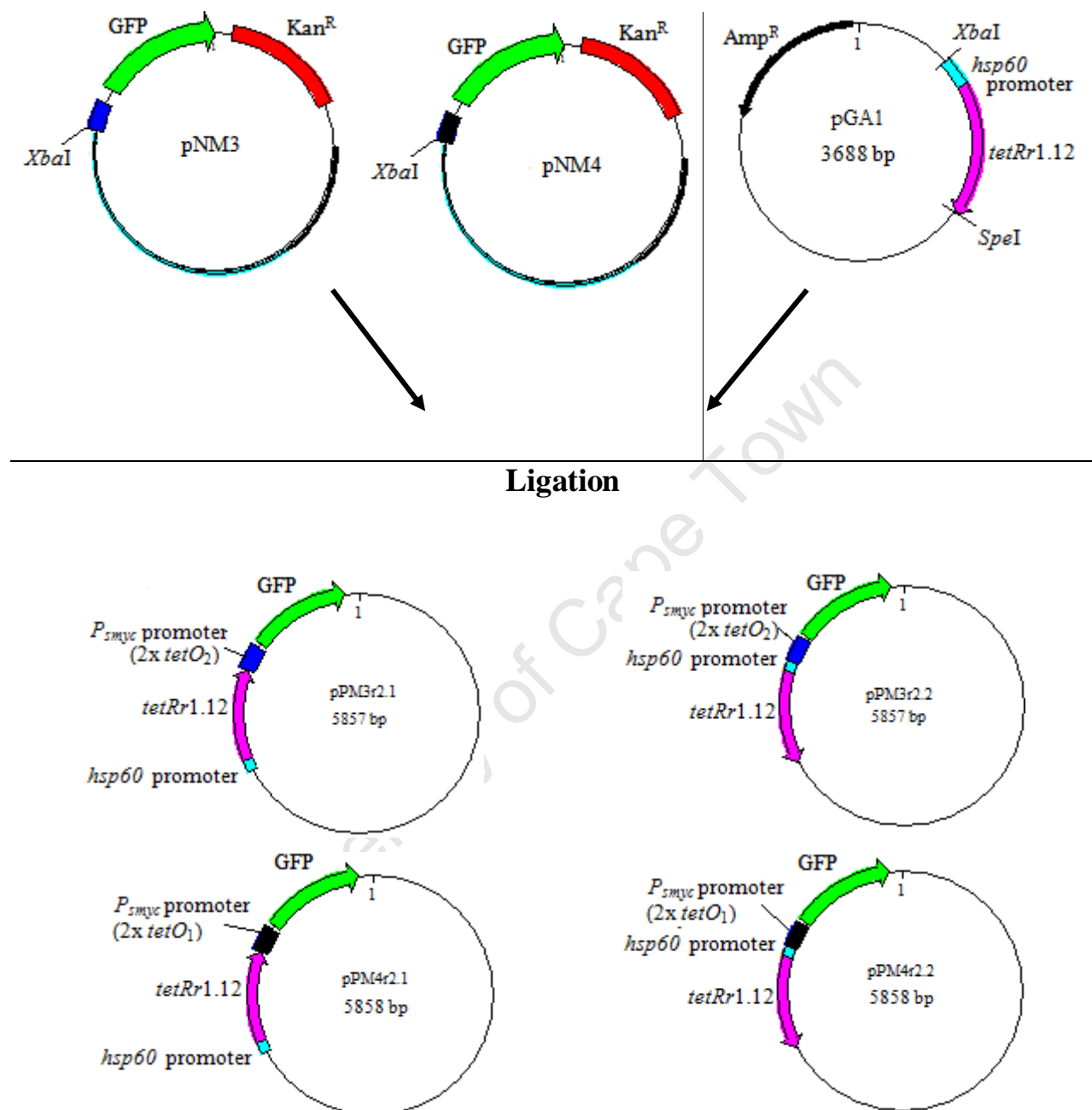


Figure 3.4: An illustration of the cloning strategy used for the generation of the TetRr1.12 shuttle plasmids. The TetRr1.12 cassette (*hsp60* promoter + *tetRr1.12*) was cleaved off from pGA1 and cloned into the pNM3 (*P_{smyc}* promoter containing 2x *tetO₂*) and pNM4 (*P_{smyc}* promoter containing 2x *tetO₁*) vectors to generate four episomal TetRr1.12 shuttle vectors.

3.2.2. CONFIRMATION AND SCREENING OF THE POSITIVE RECOMBINANTS

3.2.2.1. Restriction enzyme mapping of plasmid DNA prepared using a small-scale isolation procedure

Each ligation mix (5 µl) was transformed into *E. coli* competent cells (Appendix A3). Twenty colonies of the transformants were picked off the plates and inoculated into 800 µl of 2YT media containing the appropriate antibiotic in a 2 ml eppendorf tube. The cultures were incubated overnight standing at 37°C. Reference plates were prepared simultaneously by pricking and stabbing each selected transformant onto a numbered grid on a 2YT- kanamycin (Kan) agar plate, thus ensuring the maintenance of recombinant clones for further reference. Cultures were then subjected to a small-scale plasmid isolation procedure as described in Appendix B1.1. The plasmid DNA was digested with a combination of restriction enzymes (Table 3.1) as described in Appendix B2 and products were analysed on a 1% agarose gel (Appendix B3).

Table 3.1: Plasmid DNA mapping restriction enzymes used to confirm the recombinant plasmids

RESTRICTION ENZYMES	FUNCTION
<i>EcoRV</i>	Confirms the presence of the TetRr1.12 cassette as the site is unique to the cassette
<i>MluI</i> + <i>NotI</i>	Confirms the size change from the parental plasmids to the TetRr1.12 plasmids that contain the TetRr1.12 cassette
<i>XbaI</i> + <i>NdeI</i>	Confirms the orientation of the TetRr1.12 cassette

3.2.2.2. Restriction enzyme mapping of plasmid DNA prepared using a large-scale isolation procedure

Subsequent to plasmid DNA mapping, selected recombinants were picked off the reference plates, each was cultured in 100 ml 2YT broth with selection in a 1 L conical flask, placed on an orbital shaker at 37°C and left overnight. From each overnight culture, 5 ml was used to prepare 15% glycerol stocks that were then stored at -80°C. Plasmid DNA was extracted from the remainder of the cultures using a large-scale plasmid isolation procedure (Appendix B1.2). Finally, 500 ng - 1 µg of the plasmid DNA was subjected to restriction enzyme mapping using the enzymes listed in Table 3.1. The products were analysed on a 1% agarose gel.

3.2.3. INTRODUCTION OF THE T_{ET}RR1.12 PLASMIDS INTO MYCOBACTERIA

3.2.3.1. Preparation of mycobacterial cell starter cultures

Two mycobacterial strains were used in this study. *M. smegmatis* mc²155 (BioVac institute, Cape Town, S.A) was used as a model strain. To make vaccines we used a pantothenate auxotroph derived from *M. bovis* BCG Pasteur, *M. bovis* BCG mc²6000 (*M. bovis* BCG Δ *panCD*) obtained from Dr. William Jacobs Jr. (Albert Einstein College of Medicine, New York).

A 15% glycerol stock (0.5 ml) of mycobacterial cells stored at -80°C was thawed on ice. The aliquot was used to inoculate 5 ml of sterile MB-7H9 medium (Appendix C1) in a 50 ml conical sterile tube [Sterilin]. For *M. smegmatis* cells MB-7H9 media (Appendix C1) was used and for BCG Δ *panCD* cells MB-7H9 media containing 24 µg/ml vitamin B5 [Sigma-Aldrich] + 25 µg/ml hygromycin [Roche diagnostics] was used. The starter cultures were incubated standing with loosened lids at 37°C for approximately 2 days (*M. smegmatis*) and 5 days (BCG Δ *panCD*).

To quantify the mycobacterial cultures, a spectrophotometer [Thermo spectronic] was used. The cultures were diluted to provide an OD reading between 0.1 and 1.0 in MB-7H9 media in 2.5 ml semi-micro disposable OD cuvettes [Sterilin] and the absorbance readings were taken at a wavelength of 600 nm.

3.2.3.2. Preparation of electro-competent mycobacteria cells

The method described by Parish and Stoker (2001)²¹³ was used to prepare competent mycobacterial cells. Starter cultures were used to inoculate 100 ml of MB-7H9 media (supplemented with vitamin B5 and hygromycin for BCG Δ *panCD*) at a 1:100 dilution. When the cultures had reached an OD₆₀₀ of 0.8 units, they were incubated on ice for 1.5 hrs with slow rotation and harvested by centrifugation at 4°C, at 5000 rpm for 10 min. Cells were kept cold on ice during all subsequent procedures to enhance competency. The pellets were then washed three times in pre-chilled 10% glycerol prepared with High performance liquid chromatography (HPLC) grade H₂O [Merck]. Finally the cells were resuspended in 5 ml pre-chilled 10% glycerol representing a 20-fold concentration. The competent cells were subsequently divided into 200 µl aliquots and either transformed with plasmids immediately or stored at -80°C.

3.2.3.3. Transformation of plasmid DNA into mycobacterial cells

The procedure was performed under sterile conditions. Mycobacterial competent cells were thawed on ice and washed by centrifugation at room temperature, at 4000 rpm for 1 min. The supernatant was discarded, the cells were gently resuspended in 500 μ l ice-cold, 10% glycerol and then kept on ice. An aliquot of 40 μ l of the competent cells was mixed with plasmid DNA (500 ng–1 μ g) of the confirmed constructs and made up to a final volume of 50 μ l in 10% glycerol and transferred to a pre-chilled 0.1 mm gap cuvette [Biorad]. The competent cell mixture was then electroporated using the Gene Pulser™ [BioRad] set at Voltage: 1.8 kV, Capacitance: 25 μ F and Resistance: 1000 Ω . Time constants were noted. A laboratory stock of pCONEPI plasmid DNA (Appendix E2) was used as a positive control and 5 μ l of HPLC grade H₂O served as a negative control for transformation.

A volume of 1 ml MB-7H9 media (expression medium) (containing vitamin B5 and hygromycin B for BCG Δ panCD) was added immediately after electroporation. Transformation mixes were transferred into 20 ml tubes [Sterilin] and left standing with loosened lids at 37°C for 5 hrs for *M. smegmatis* and overnight for BCG Δ panCD. Cultures were plated on the appropriate selective MB-7H10-OADC agar containing appropriate supplements for BCG Δ panCD. Plates were sealed in plastic bags to prevent dehydration and incubated at 37°C for 3 days for r*M. smegmatis* and 3 - 5 weeks for rBCG Δ panCD.

3.2.3.4. Preparation of stocks of recombinant mycobacteria

a. Glycerol stocks

Mycobacterial colonies were picked off the plates and dispersed in 5 ml of MB-7H9 media containing appropriate supplements by a gentle pipeting action to make starter cultures. The rBCG Δ panCD colonies were firstly broken apart manually using sterile toothpicks before dispersing into 5 ml media. After significant growth was observed, the starters were swirled, the larger clumps allowed to settle briefly and the top homogenous layer used to inoculate 10 ml of MB-7H9 media in order to eliminate the presence of bacterial clumps in the cultures. When the 10 ml cultures reached OD₆₀₀ of approximately 0.8 units, they were used to inoculate 100 ml of the same media in 1 L Schott bottles. The cultures were incubated standing at 37°C for 1 day for the r*M. smegmatis* and 3 - 4 days for rBCG Δ panCD, then placed on slowly turning rollers.

These cultures were grown to an OD₆₀₀ of approximately 0.5 units and used to prepared 15% glycerol stocks, which were stored at -80°C.

b. Vaccine stocks

The starter cultures were prepared as described in Section 3.2.3.1, in addition 10 µg/ml Kan was included for plasmid selection. Once the starter cultures had reached OD₆₀₀ of 0.8 units, they were used to inoculate 100 ml of the same media in 1 L Schott bottles. The cultures were incubated standing at 37°C for 1 day for the r*M. smegmatis* and 3 - 4 days for rBCG Δ *panCD*, then placed on slowly turning rollers until they reached an OD₆₀₀ of 0.5 units. A volume of 10 ml was taken from each culture and used to prepare 15% glycerol stocks and stored at -80°C. The remaining cultures were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The pellets were resuspended in resuspension buffer (Appendix C3) to an OD₆₀₀ of 10 to make vaccine stocks. The vaccine stocks were subsequently divided into 200 µl aliquots in 2 ml cryovials [Greiner Bio-one], which were frozen at -80°C.

3.2.3.5. Evaluation of genetic integrity of the plasmid DNA from the recombinant mycobacteria vaccine stocks

The BCG Δ *panCD* or *M. smegmatis* vaccine stocks (200 µl) were thawed and then precipitated at 14 000 rpm for 2 min at room temperature using the 5417C bench-top centrifuge [Eppendorf]. The pellets were resuspended in 400 µl GTE-lysozyme buffer (Appendix C3) and incubated with slight shaking at 37°C for 3 hrs. Plasmid DNA was then isolated from the cell suspensions using the Qiaprep® Spin miniprep kit [Qiagen] according to manufacturer's instructions. The isolated plasmid DNA was transformed into *E. coli* as described in Appendix A3. Twenty colonies of the transformants were picked off the plates, the plasmid DNA was isolated using small-scale isolation procedure as described in section 3.2.2.1 and subjected to restriction enzyme mapping as described in Section 3.2.2.1.

3.2.4. A PILOT ASSESSMENT OF THE TETRR1.12 SYSTEMS IN *M. smegmatis* USING ANHYDROTETRACYCLINE: PROOF OF THE CONCEPT

Mayat, (2009)²⁶⁴ established the minimum inhibitory concentration (MIC) of ATc for *M. smegmatis*, i.e. the minimum concentration of ATc that inhibits bacterial growth. She reported that *M. smegmatis* growth was inhibited at 400 ng/ml ATc. Therefore in this study we used ATc at < 400 ng/ml for the pilot experiment.

Duplicate starter cultures of *rM. smegmatis* harbouring the TetRr1.12 plasmids, parental plasmids pNM3 and pNM4 (positive controls) and the pCONEPI (negative control) (Appendix E2) were prepared as described in section 3.2.3.1. One culture was grown in media containing 10 µg/ml Kan and 250 ng/ml ATc and the other culture grown in media containing only 10 µg/ml Kan. Due to light sensitivity of ATc, the cultures were incubated in the dark and protected from light during handling. From each starter culture, 5 ml was inoculated into 100 ml of the same media in a 1 L Schott bottle and placed on rollers at 37°C. At OD₆₀₀ of 0.5 units, 30 ml from each culture was subjected to a fluorimetry assay (Appendix B7.1).

To assess induction, ATc was washed from the remainder of the cultures by centrifugation at 4000 rpm for 10 min at 4°C, the pellets were resuspended in 70 ml of the same media (without ATc) and incubated at 37°C on rollers. One hour later, 30 ml was taken from the cultures and assayed by fluorimetry. The remainder of the cultures were incubated at 37°C on rollers for a further 24 hrs and subsequently assayed as before.

3.2.5. ASSESSMENT OF THE *rM. smegmatis*[TetRr1.12] SYSTEMS

3.2.5.1. Minimum inhibitory concentration of anhydrotetracycline required to repress the *rM. smegmatis*[TetRr1.12] systems

The first aim was to determine the optimal ATc concentration for each of the *rM. smegmatis*, that is the minimum concentration of ATc that would inhibit the expression of GFP. The second aim was to, (i) repress the GFP expression by adding ATc into the cultures and note the repression intensity, (ii) remove ATc and monitor the induction, and (iii) note the times at which the highest levels of GFP were achieved. The findings from experiment 3.2.4 were used to estimate the ATc concentration ranges to use. The constructs that were not completely inhibited by 250 ng/ml ATc were subjected to a higher ATc concentration range (200 - 400 ng/ml). The constructs that were completely inhibited by 250 ng/ml ATc, were subjected to a lower ATc range (90 - 250 ng/ml).

rM. smegmatis starter cultures containing the TetRr1.12 plasmids were prepared as described in Section 3.2.3.1. One culture was grown in ATc-free MB-7H9 media and the rest were grown in MB-7H9 media containing varying amounts of ATc (Table 3.2). *rM. smegmatis* containing the positive control pNM3 and negative control pCONEPI were grown in MB-7H9 media containing 250 ng/ml ATc. The cultures were grown standing in the dark at 37°C and then used to inoculate

100 ml of the same MB-7H9 media in 1 L Schott bottles and grown on rollers at 37°C to a high OD₆₀₀ of 0.8. A volume of 10 ml from each culture was assayed by fluorimetry, and 100 µl was plated on MB-7H10-Kan agar plates containing the same concentrations of ATc. The plates were covered with foil to protect them from the direct light, wrapped in plastic sandwich bags to prevent dehydration and incubated at 37°C for 3 - 4 days. The colonies were visualized over a UV trans-illuminator Chromato-vue TM-20 Trans-illuminator [UVP] at 245 nm (UV range) and photos were taken at various exposures (-0.1s - +0.1s) using a digital camera [Nikon 4500]. The images were used to compare the fluorescence levels displayed by the colonies produced on MB-7H10-Kan agar containing ATc at different concentrations.

Table 2.2: The concentration ranges of anhydrotetracycline used for the *rM. smegmatis*[TetRr1.12] systems. *rM. smegmatis* were cultured with the following ATc concentrations in order to determine the optimal ATc concentration for each system.

	PLASMID NAMES	ATC CONCENTRATION (ng/ml)
Group 1	pPM3r2.1	250 - 400
	pPM3r2.2	90 - 250
Group 2	pPM4r2.1	200 - 350
	pPM4r2.2	90 - 250
Group 3	pNM3	250
	pNM4	250
	pCONEPI	250

GFP expression was induced by removing ATc from the remaining cultures. The cultures were centrifuged at 4000 rpm for 15 min, and the pellets were resuspended in fresh MB-7H9 media and centrifuged as before. The pellets were then resuspended in 100 ml fresh MB-7H9-Kan media, one in media containing ATc and the other in media lacking ATc. The cultures were placed back on rollers at 37°C and subsequently assayed by fluorimetry, at 12 hr intervals for a period of 72 hours in order to monitor the induction of GFP expression.

3.2.5.2. Comparison of genetic integrity of the plasmid DNA isolated from *rM. smegmatis* cultured in the presence and absence of ATc

The optimal ATc concentrations derived in the previous section were used for this experiment. Duplicate starter cultures of *rM. smegmatis* were prepared as described in Section 3.2.3.1, one

culture was grown in media containing ATc and the other culture was grown in media lacking ATc. ATc was then removed from the cultures as described in the previous section to induce the cultures. Seventy-two hours after ATc removal, plasmid DNA was isolated from the *rM. smegmatis* cultures, the genetic integrity of the plasmids was evaluated as described in section 3.2.3.5 and the percentages of stable clones amongst the selected clones was calculated.

3.2.5.3. Genetic stability of the recombinant plasmids in the model organism *M. smegmatis*

Starter cultures of the *rM. smegmatis* harbouring the TetRr1.12 plasmids and the control plasmids, pNM3 (positive control), and pCONEPI (negative control) were prepared in triplicate as described in Section 3.2.3.1. At an OD₆₀₀ of 0.8 units the starter cultures were used to inoculate 100 ml of the MB-7H9 media containing different amounts of Kan and ATc in 1 L Schott bottles (cultures were duplicated): the first contained no Kan and no ATc (Kan⁻ ATc⁻), the second had Kan (10 µg/ ml) and no ATc (Kan⁺ ATc⁻) and the third contained both Kan (10 µg/ ml) and ATc at optimal concentrations (Kan⁺ ATc⁺). The cultures were grown at 37°C on rollers. At an OD₆₀₀ of approximately 0.5 units, each culture was treated according to the following steps:

1. 1 ml was quantified with the spectrophotometer to obtain an accurate OD₆₀₀ reading.
2. 1 ml was diluted in resuspension buffer (Appendix C3) to 10⁻⁵, 10⁻⁶ and 10⁻⁷ (the dilutions were duplicated), plated on MB-7H10 agar plates containing different amounts of Kan and ATc (as described above) and incubated for 3 - 4 days at 37°C.
3. 500 µl was used to inoculate 50 ml of fresh MB-7H9-Kan medium.
4. The remaining culture was centrifuged at 4000 rpm for 10 min, resuspended in resuspension buffer to an OD of 10 units and stored at -80°C.

The above steps were repeated 6 times in order to passage the cultures for approximately ± 42 generations. Based on the recorded optical densities, the approximate generation of each culture was calculated according to the following equation: Generation = [ln (final OD₆₀₀) - ln (starting OD₆₀₀)] / ln 2²⁶⁶. Colonies were visualized over a UV trans-illuminator at 245 nm (UV range) and photos were taken at various exposures using a digital camera. Colony sizes were noted and the percentage of colony forming units which maintained GFP fluorescence was calculated. Colony counts from plates with and without Kan selection were also used to calculate the percentage of colony forming units (cfu) that retained antibiotic resistance. Genetic stability of

rM. smegmatis was taken as a measure of the number of colonies that fluoresced after \pm 40 generations.

3.2.6. INTRODUCTION OF THE TETR1.12 REGULATORY SYSTEM INTO *M. bovis* BCG Δ PANCD

A pantothenate auxotrophic strain *M. bovis* BCG mc² 6000 (BCG Δ panCD) created by Bardarov *et al.*, (2002)²⁶⁷ was used as a host strain for recombinant vaccine vectors.

3.2.6.1. Evaluation of the rBCG Δ panCD harbouring the TetRr1.12 plasmids in the presence of anhydrotetracycline

The TetRr1.12 plasmids were electro-transformed into BCG Δ panCD. rBCG Δ panCD responsiveness to ATc was then assessed in order to estimate the concentration range of ATc suitable for the regulation of GFP expression. Duplicate starter cultures of the recombinants, rBCG Δ panCD[TetRr1.12], rBCG Δ panCD[pNM4] positive control and rBCG Δ panCD[pCONEPI] negative control were prepared as described in Section 3.2.3.1, in addition 10 μ g/ml Kan was included for plasmid selection. One culture was grown in ATc-free MB-7H9 media and the rest were grown in MB-7H9 media containing varying amounts of ATc, 1.5 - 3.0 μ g/ml. The cultures were grown in the dark at 37°C and at an OD₆₀₀ of 0.8 units, 5 ml from each culture was inoculated into 100 ml of the same MB-7H9 media in 1 L Schott bottles. The recombinant cultures were grown on rollers at 37°C to a high OD₆₀₀ of 0.8. From each culture, a volume of 10 ml was assayed by fluorimetry and 100 μ l was plated on MB-7H10-Kan agar plates containing similar varying concentrations of ATc. The plates were covered, wrapped in plastic sandwich bags and incubated at 37°C. To note the difference in fluorescence, 3 - 4 weeks later the colonies were visualized over a UV trans-illuminator at 245 nm and photographs captured using a digital camera.

The remaining cultures were washed in order to induce the expression of GFP by centrifuging at 4000 rpm for 10 min, resuspending the cell pellets in 70 ml of ATc-free media and centrifuging as before. Finally the pellets were resuspended in 70 ml of ATc-free media and incubated at 37°C on rollers. On a weekly basis for a period of 3 weeks, 20 ml from each culture was analysed using fluorimetry and the remaining culture was placed back on rollers at 37°C.

3.2.6.2. Assessment of TetRr1.12 plasmid stability in BCG $\Delta panCD$

The aim of this experiment was to assess the genetic stability of the recombinant plasmids in the presence and absence of the ATc in BCG $\Delta panCD$. An ATc concentration of 2 $\mu\text{g/ml}$ was used for the experiment since this was the optimal concentration for all the constructs (established in the previous section). rBCG $\Delta panCD$ starter cultures harbouring the TetRr1.12 and the controls plasmids, pNM4 and pCONEPI were prepared in duplicate as described in Section 3.2.3.1, in addition 10 $\mu\text{g/ml}$ Kan was included for plasmid selection. One culture was grown in the presence of ATc and the other was grown without ATc. The starter cultures were subsequently used to inoculate 100 ml of the same media and propagated at 37°C on rollers. At an OD₆₀₀ of approximately 0.5 units, the cultures were processed according to the steps in section 3.2.6, in addition, 20 ml of each culture was subjected to a fluorimetry assay. Genetic integrity of plasmid DNA isolated from rBCG $\Delta panCD$ cultures produced after the final passage was assessed as described in section 3.2.3.5.

3.3. RESULTS

3.3.1. CONSTRUCTION OF THE TetRr1.12 SHUTTLE PLASMIDS

To investigate the TetRr1.12 regulatory system, four different versions of the shuttle plasmid were generated, by cloning the TetRr1.12 expression cassette into the parental pNM3 and pNM4 plasmids that were generated by Mayat, (2009)²⁶⁴ (as demonstrated in Figure 3.4). Restriction enzyme mapping was then performed to screen for potentially positive recombinants, where small-scale plasmid DNA isolated from *E. coli* clones transformed with the various ligation mixes was subjected to restriction enzyme digestion. An *EcoRV* digest was performed to confirm the presence of the TetRr1.12 cassette since the site is unique to the cassette, the double digest *MluI* + *NotI* was performed to confirm the presence of the TetRr1.12 cassette as it showed the size change from the parental plasmids to the TetRr1.12 plasmids, and finally the double digest *XbaI* + *NdeI* was performed to confirm the orientation of the TetRr1.12 cassette (the maps shown in Figure 3.5).

Mapping results showed that the *tetRr1.12* repressor gene had inserted successfully immediately upstream of the strong *P_{smyc}* promoter in two different orientations with respect to the *gfp* gene, as shown in Figure 3.5. The constructs are listed in Table 3.3.

Table 3.3: Description of *E.coli*/mycobacterial shuttle vectors containing the TetRr1.12 expression cassette. pNM3 contain the full-length P_{smyc} promoter with two $tetO_2$ sequences situated on either side of -10 and pNM4 contain the full-length P_{smyc} promoter with two $tetO_1$ sequences situated on either side of -1.

PLASMID NAME	FEATURES
pPM3r2.1	pNM3 containing the TetRr1.12 cassette in the same orientation as the <i>gfp</i> gene
pPM3r2.2	pNM3 containing the TetRr1.12 cassette in the opposite orientation to the <i>gfp</i> gene
pPM4r2.1	pNM4 containing the TetRr1.12 cassette in the same orientation as the <i>gfp</i> gene
pPM4r2.2	pNM4 containing the TetRr1.12 cassette in the opposite orientation to the <i>gfp</i> gene

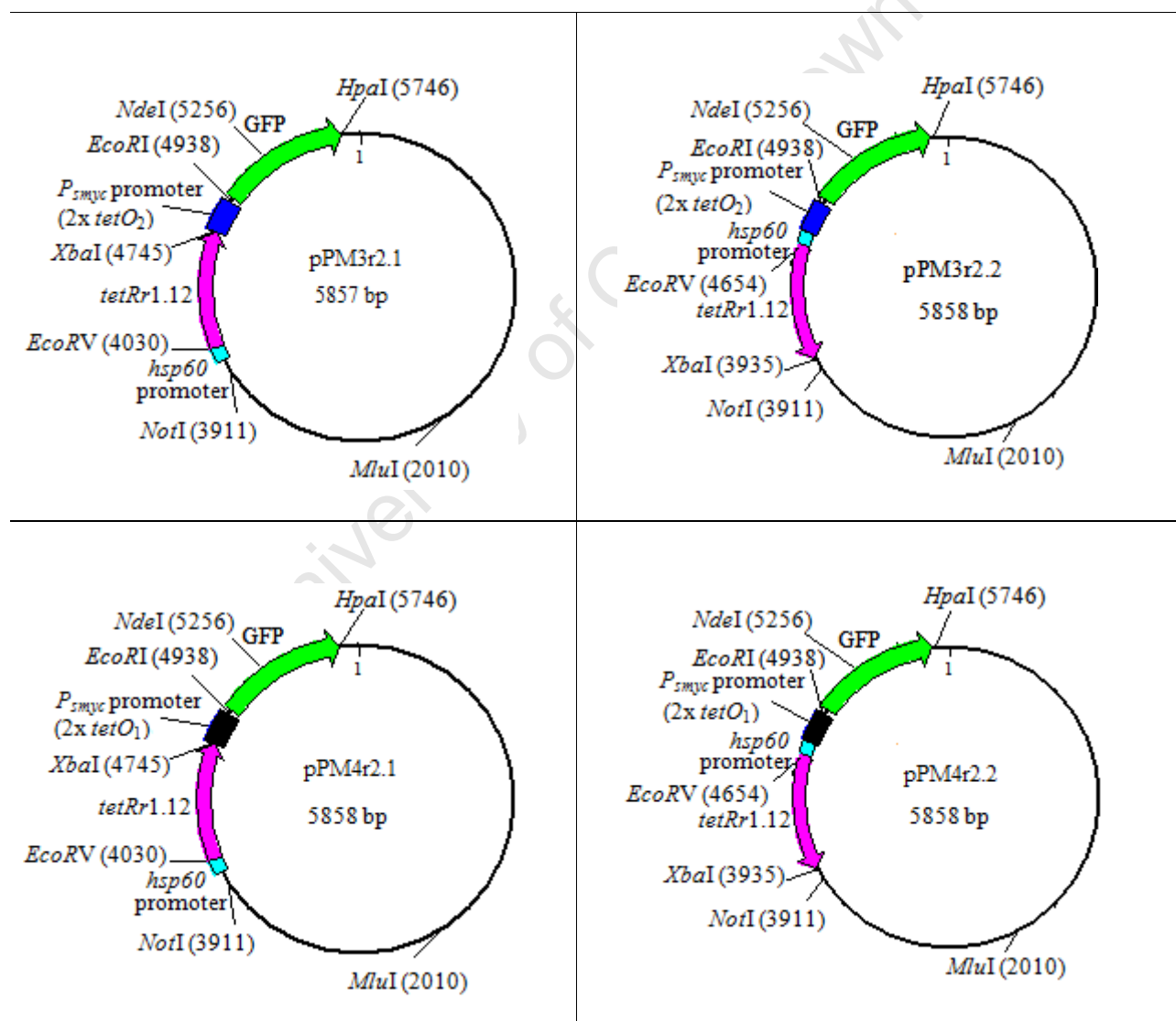


Figure 3.5: The maps of the episomal TetRr1.12 dependant regulatory system designs. These plasmids were constructed by inserting the TetRr1.12 cassette into the pNM3 and pNM4 vector backbones.

3.3.2. TRANSFORMATION OF TetRr1.12 SHUTTLE PLASMIDS INTO MYCOBACTERIA

3.3.2.1. Introduction of the TetRr1.12 plasmids into *M. smegmatis*

The confirmed TetRr1.12 plasmids were successfully introduced into the model strain *M. smegmatis* by electroporation. This is fast growing, non-pathogenic mycobacteria that has been widely used as a model organism to study the biology of other virulent strains and extremely slow growing species of mycobacteria²⁶⁸. *rM. smegmatis* stocks from the electro-transformants were prepared. To verify the genetic integrity of the recombinant *M. smegmatis* plasmids, plasmid DNA was isolated from the stocks and subjected to restriction enzyme mapping using the same enzymes shown in Table 3.1. The results showed that all four *E.coli*/mycobacterial shuttle vectors containing the TetRr1.12 cassette remained intact in *M. smegmatis*.

3.3.2.2. A Pilot experiment performed to test the responsiveness of *rM. smegmatis* to anhydrotetracycline

This pilot experiment was designed to assess whether GFP expression could be repressed in the presence of ATc and induced upon its removal. As mentioned in Chapter 2, the affinity of ATc for the repressors is significantly higher than its affinity for the 30S ribosomal subunit of the host, thus ATc levels required for protein inhibition are considerably higher than the levels required to repress the expression of the recombinant antigen^{249, 251}. Mayat, (2009)²⁶⁴ reported that 400 ng/ml ATc inhibits *M. smegmatis* growth, therefore ATc concentration of 250 ng/ml was used.

The amount of GFP expression in bacterial lysates prepared from *rM. smegmatis* cultures harbouring the recombinant plasmids was measured by a fluorimetry assay. In 2009, Mayat²⁶⁴ established the optimal cell culture density to be an OD₆₀₀ of 2 units for fluorimetry experiments, which was the similar to that reported by Carroll and Parish in 2005²⁶⁹, and was therefore used in this study. The parentals, *rM. smegmatis*[pNM3] and *rM. smegmatis*[pNM4] that lack the TetRr1.12 cassette were the positive controls as these are not affected by ATc. *rM. smegmatis*[pCONEPI] lacking the *gfp* gene was the negative control and accounted for the background readings, which were subtracted from the experimental sample readings.

A comparison was made between the recombinants that were cultured with ATc and those that were cultured without ATc, in terms of the levels of GFP expression. As shown in Figure 3.6, it is clear that the TetRr1.12 cassette inhibited GFP expression in the presence of ATc as all the *rM. smegmatis* showed low fluorimetry readings as compared to the *rM. smegmatis* grown without ATc. However, *rM. smegmatis* appeared to be partially repressed since the levels of GFP were not as low as the background readings. *rM. smegmatis* harbouring pPM3r2.2 and pPM4r2.2 were more repressed than *rM. smegmatis* harbouring pPM3r2.1 and pPM4r2.1.

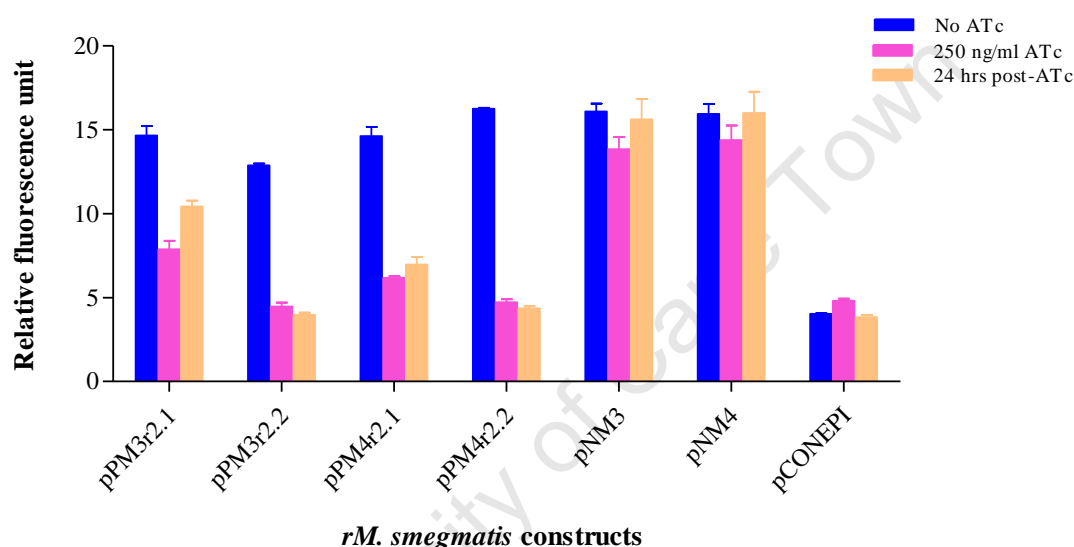


Figure 3.6: A fluorimetry assay showing the ATc-dependent repression of GFP expression in the *rM. smegmatis* and corresponding induction upon ATc removal. GFP expressed by the cultures grown in the absence of ATc (blue bars), in the presence of 250 ng/ml ATc (pink bars) and readings recorded 24 hrs after ATc removal (orange bars). The positive controls were pNM3 and pNM4, and negative control was pCONEPI. Error bars represent the standard deviations from two experiments.

ATc was then removed from the recombinant cultures and 24 hrs later the induction was assessed, shown by orange bars in Figure 3.6. *rM. smegmatis* harbouring the plasmids pPM3r2.1 and pPM4r2.1 were slightly induced, whereas no induction could be seen from *rM. smegmatis* harbouring the plasmids pPM3r2.2 and pPM4r2.2. This data indicates that *rM. smegmatis*[pPM3r2.2] and *rM. smegmatis*[pPM4r2.2] were more sensitive to ATc.

Overall, the experiment demonstrated that the TetRr1.12 regulatory system could repress the expression of the recombinant antigen in *M. smegmatis* in the presence of ATc. However, the different *rM. smegmatis* systems displayed different levels of sensitivity to ATc. The systems containing the TetRr1.12 cassette in the opposite orientation to the *gfp* gene (*rM. smegmatis*[pPM3r2.2] and *rM. smegmatis*[pPM4r2.2]) were very sensitive to ATc, whereas those containing the cassette in the same orientation as the *gfp* gene (*rM. smegmatis*[pPM3r2.1] and *rM. smegmatis*[pPM4r2.1]) were less sensitive. The background readings produced by the negative controls could be attributed to fluorescence of mycobacterial proteins in the cultures.

3.3.2.3. Determination of the concentration of anhydrotetracycline needed to regulate GFP expression in *rM. smegmatis*[TetRr1.12] systems

Having established that the TetRr1.12 regulatory systems displayed dissimilar sensitivity levels to ATc, the next task was to determine the minimum concentration of ATc that would inhibit GFP expression, and easily be removed to permit induction. The findings from the pilot experiment were used to estimate the ATc concentration range to use in this experiment. The higher range, 200 - 400 ng/ml was used for the less sensitive systems, *rM. smegmatis*[pPM3r2.1] and *rM. smegmatis*[pPM4r2.1], and the lower ATc range, 90 - 250 ng/ml was used for more sensitive systems, *rM. smegmatis*[pPM3r2.2] and *rM. smegmatis*[pPM4r2.2]. Recombinant colony fluorescence was compared by visualization over a UV trans-illuminator.

rM. smegmatis colonies are shown in Figure 3.7. Panel A shows the *rM. smegmatis* controls, as you would expect the positive controls, *rM. smegmatis*[pNM3] and *rM. smegmatis*[pNM4] displayed good fluorescence, and the negative control, *rM. smegmatis*[pCONEPI] did not fluoresce at all. Shown in panels B - E are the recombinant colonies exhibiting different fluorescence intensities in the presence of varying amounts of ATc. The minimum concentrations of ATc that achieved GFP repression were selected as the optimal concentrations, these were 250 ng/ml for *rM. smegmatis*[pPM3r2.1], 100 ng/ml for *rM. smegmatis*[pPM3r2.2], 200 ng/ml for *rM. smegmatis*[pPM4r2.1] and 100 ng/ml for *rM. smegmatis*[pNM4r2.2].

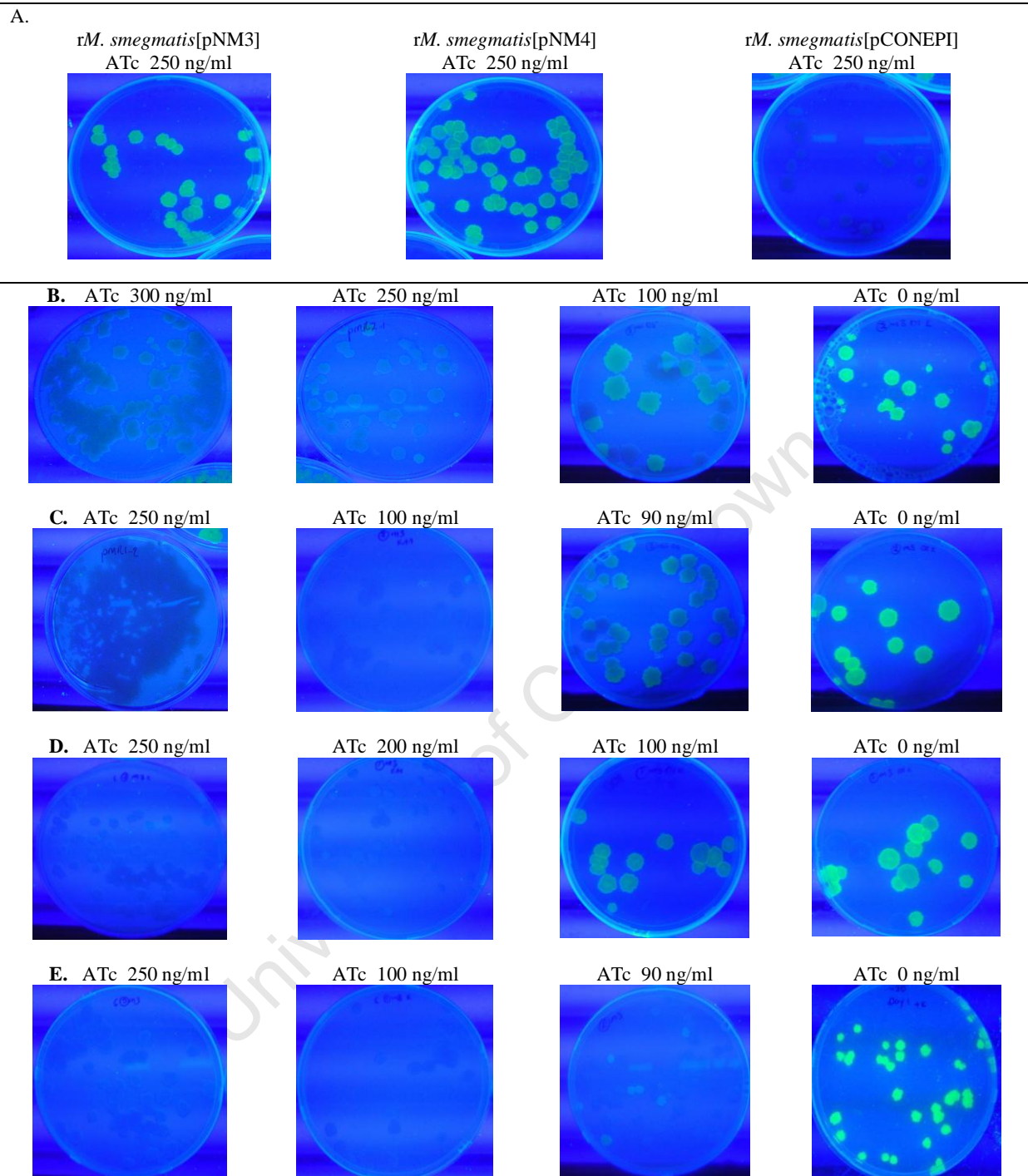


Figure 3.7: Images taken over UV light illustrating ATc-dependant repression of GFP expression of the *rM. smegmatis* systems. *rM. smegmatis*[TetRr1.12] systems were exposed to different ATc concentration ranges in order to determine the minimum concentration of ATc that would inhibit GFP expression. A. Positive controls, *rM. smegmatis*[pNM3] and *rM. smegmatis*[pNM4] and negative control, *rM. smegmatis*[pCONEPI]; B. *rM. smegmatis*[pPM3r2.1]; C. *rM. smegmatis*[pPM3r2.2]; D. *rM. smegmatis*[pPM4r2.1] and E. *rM. smegmatis*[pPM4r2.2]

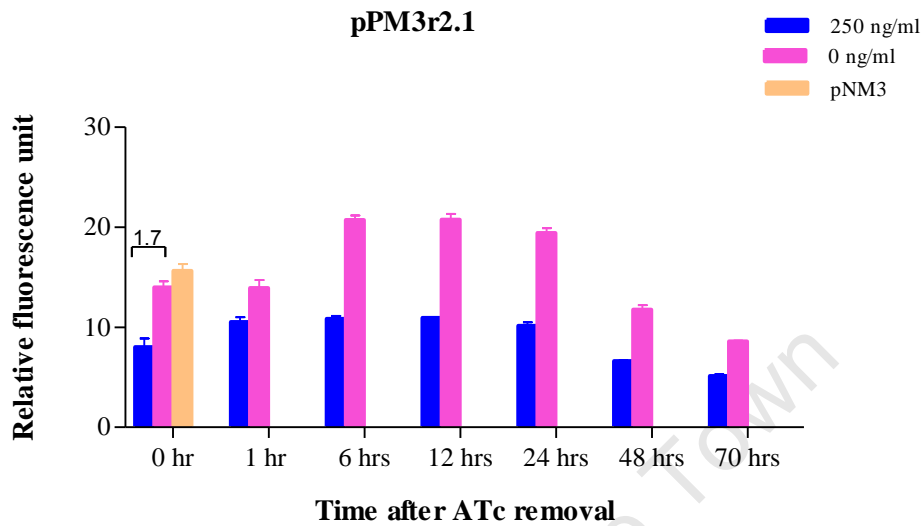
The following experiment was performed to confirm whether the repression achieved with the optimal ATc levels could be removed and allow induction to occur, to determine the time point at which the highest GFP levels were achieved and to compare GFP levels produced from *rM. smegmatis* that were initially repressed to those that were not repressed. Two batches of each *rM. smegmatis* were prepared, one was grown with ATc at optimal concentrations and the other without ATc. The recombinant cultures were then washed to remove ATc and induce GFP expression and the induction kinetics were monitored for a ± 70 hr period by the whole cell fluorimetry assay.

The results can be seen in Figures 3.8 and 3.9, at time zero, *rM. smegmatis* that were cultured with ATc were still repressed, as the readings were very low compared to those of *rM. smegmatis* grown without ATc. Repression was approximately 1.7-fold for *rM. smegmatis*[pPM3r2.1], 1.4-fold for *rM. smegmatis*[pPM4r2.1] and 2.5-fold *rM. smegmatis*[pPM3r2.2] and *rM. smegmatis*[pNM4r2.2].

Slow induction was observed with time and the highest readings were obtained between 12 - 24 hrs. The GFP expression levels achieved from *rM. smegmatis* grown with ATc did not reach the levels of those achieved by *rM. smegmatis* grown without ATc, even after a 70/72 hr induction period. This indicates that maximal induction was not achieved after ATc was removed from the *rM. smegmatis* cultures. A positive correlation was observed between GFP expression and the growth phase of the recombinants.

The optimal levels of ATc were unable to completely repress the *rM. smegmatis* systems as the fluorimetry readings were above the background readings (Figure 3.8 - 3.9), however, the amount of ATc could not be increased since ATc above optimal levels was very difficult to remove during induction (as shown in the pilot experiment, Figure 3.6). Moreover, as can be seen above *rM. smegmatis* systems did not show complete induction after ATc was removed, yet ATc could not be reduced since ATc levels below the optimal levels were unable to repress GFP expression (Figure 3.7).

A.



B.

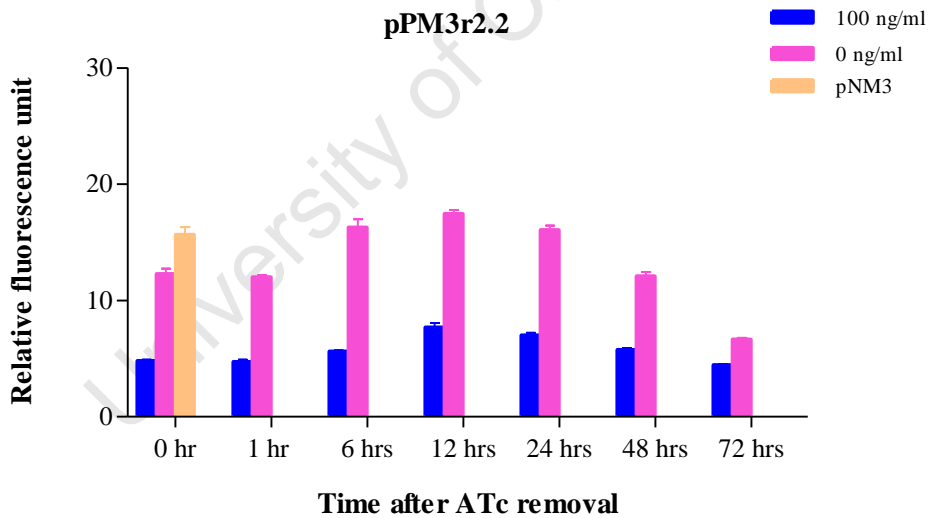
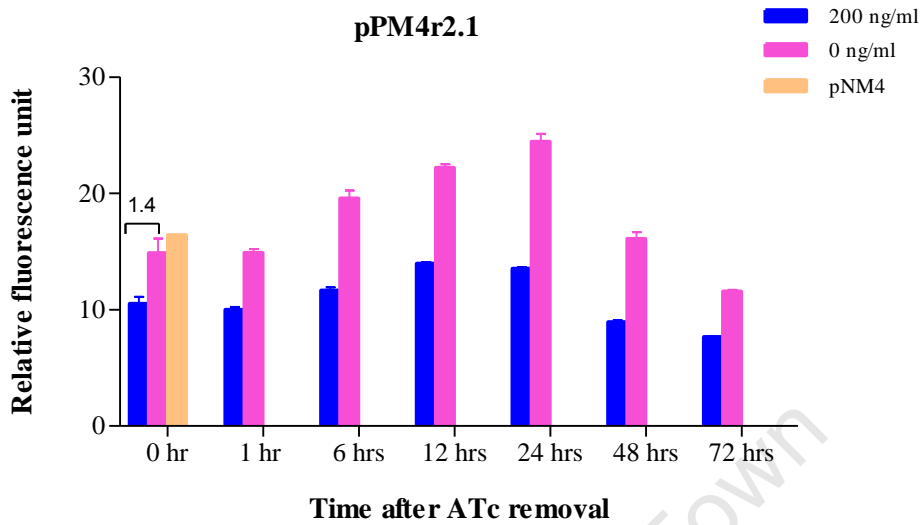


Figure 3.8: Relative GFP fluorescence of the cultures, *rM. smegmatis*[pPM3r2.1] and *rM. smegmatis*[pPM3r2.2] measured after ATc was removed. GFP expression was measured using the fluorimetry assay and expressed as relative fluorescence units after subtracting the background readings of *rM. smegmatis*[pCONEPI]. Represented by blue bars are the readings obtained from *rM. smegmatis* lysates after the removal of ATc, the pink bars represent the readings produced by *rM. smegmatis* grown in its absence, the positive control, *rM. smegmatis*[pNM3] is represented by an orange bar. The error bars represent the standard deviations from two experiments.

A.



B.

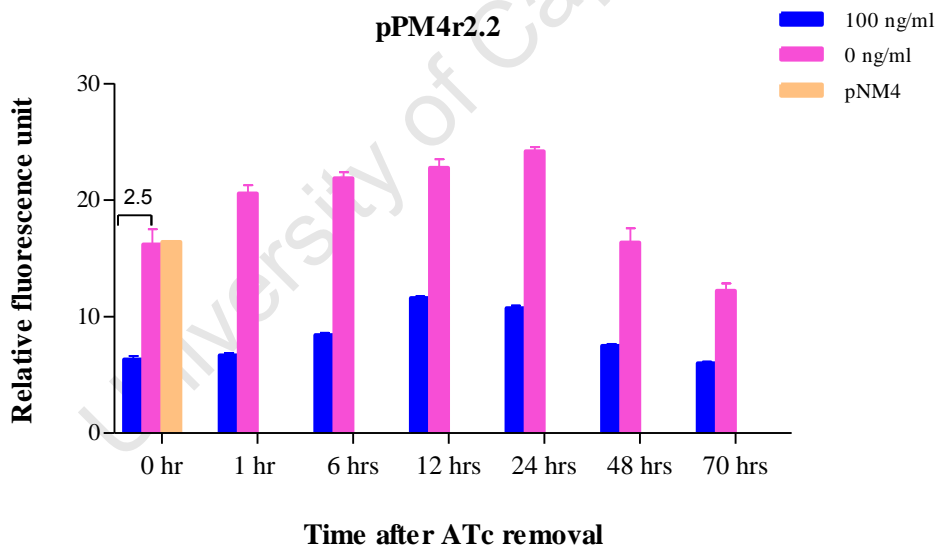


Figure 3.9: Relative GFP fluorescence of the cultures, *rM. smegmatis*[pPM4r2.1] and *rM. smegmatis*[pPM4r2.2] measured after ATc was removed. GFP expression was measured using the fluorimetry assay and expressed as relative fluorescence units after subtracting the background readings of *rM. smegmatis*[pCONEPI]. Represented by blue bars are the readings obtained from *rM. smegmatis* lysates after the removal of ATc, the pink bars represent readings produced by *rM. smegmatis* grown in its absence, the positive control, *rM. smegmatis*[pNM4] is represented by an orange bar. The error bars represent the standard deviations from two experiments.

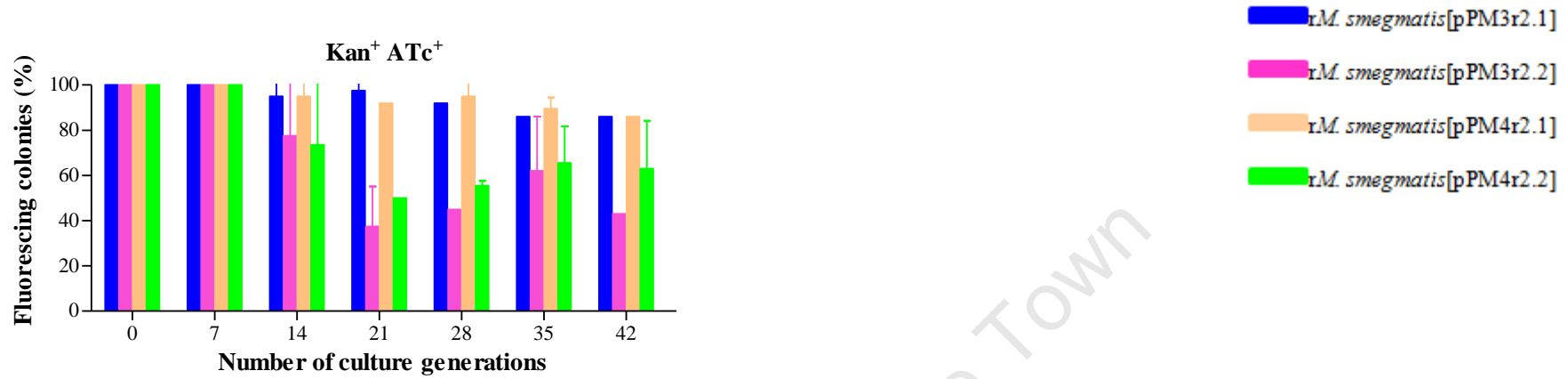
Plasmid instability have been reported in many studies in recombinant mycobacteria utilising episomal vectors and constitutive promoters, whereby the recombinant antigen is lost^{167, 270}. Hence in this study *rM. smegmatis* cultured without regulation were expected to become unstable overtime and produce lower levels of GFP. However that was not the case, since *rM. smegmatis* cultured without ATc showed higher levels of GFP than the systems that were grown with ATc (theoretically more stable), despite inducing for ± 72 hrs. Thus we assessed whether the differences in GFP expression levels correlated to plasmid instability by evaluating the genetic integrity of plasmid DNA isolated from the cultures 70/72 hrs post induction using restriction enzyme mapping.

A total of 20 clones were mapped for each *rM. smegmatis*. The results showed that *rM. smegmatis* grown without ATc were genetically unstable despite high GFP expression, since the percentages of stable clones were 50% for *rM. smegmatis*[pPM3r2.1], 35% for *rM. smegmatis*[pPM3r2.2], 46% for *rM. smegmatis*[pPM4r2.1] and 38% for *rM. smegmatis*[pNM4r2.2] (data not shown). As could be seen in the Figures 3.8 and 3.9, the levels of GFP started decreasing after 24 hours, we suspect that vector instability took place during these time points. In contrast, plasmid DNA isolated from *rM. smegmatis* that were grown with ATc and then induced for ± 72 hours were genetically stable as these produced the expected band patterns following restriction enzyme mapping, i.e. 100% of the mapped clones were stable (data not shown).

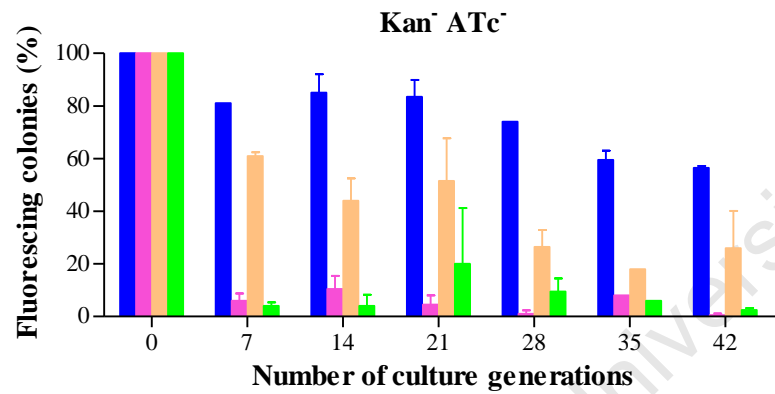
3.3.2.4. Assessment of the stability of the TetRr1.12 plasmids in *M. smegmatis*

This experiment was performed to evaluate the fitness of the TetRr1.12 vectors in *M. smegmatis* over 42 generations with or without ATc and antibiotic selection. This was done to assess whether regulating the recombinant antigen expression improves the plasmid stability and to determine the percentage of plasmid loss. At each passage (after ≈ 7 generations) maintenance of GFP fluorescence by the recombinants was assessed and the retention of Kan resistance was determined. The summary of the results can be seen in Figures 3.10. Panel A shows the results obtained in the presence of both ATc and Kan. *rM. smegmatis* systems were reasonably stable after 14 generations as the percentages of fluorescing colonies were $> 70\%$, however instability increased after the 21st generation and the percentages of fluorescing colonies ended up between 45 and 86% after 42 generations. Panels B and C show colonies produced in the absence of both

A.



B.



C.

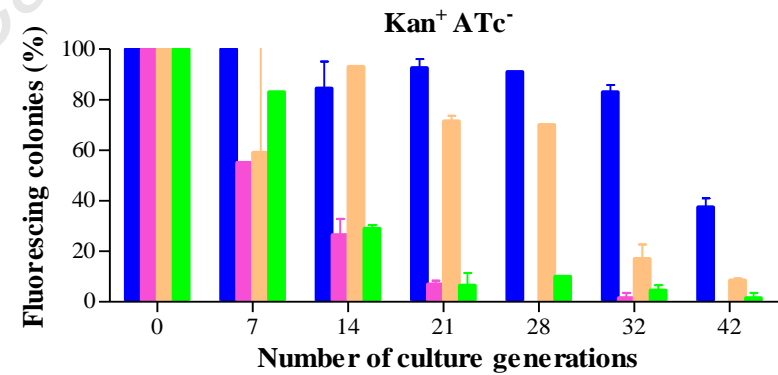


Figure 3.10: Genetic stability of the *rM. smegmatis*[TetRr1.12]. GFP expressed by the constructs was assessed in solid medium in the presence of ATc and Kan over 42 generations. Colonies were viewed over UV light and the percentage of fluorescing colonies was noted. **A.** Kan⁺ ATc⁺, **B.** Kan⁻ ATc⁻ media, and **C.** Kan⁺ ATc⁻ media. Error bars represent the standard deviations from two experiments.

Kan and ATc and in the presence of Kan and absence of ATc, respectively. As can be seen in the figures instability started occurring after the seventh generation in all the recombinant systems. This data indicates that plasmid instability occurred in the recombinant systems over time, however it occurred faster in the absence of Kan than in its presence. *rM. smegmatis*[pPM3r2.1] and *rM. smegmatis*[pPM4r2.1] appeared to be more stable than *rM. smegmatis*[pPM3r2.2] and *rM. smegmatis*[pPM4r2.2] as they produced a higher percentage of fluorescing colonies after 42 generations in the presence as well as absence of ATc.

Plasmid stability was also measured by evaluating the colony counts produced by the recombinants with and without antibiotic selection over many generations. Duplicate cultures of *rM. smegmatis* were passaged without selection over 42 generations. At each passage, the cultures were plated onto media containing and lacking kanamycin. Retention of kanamycin resistance was determined by calculating the colony counts from media containing kanamycin as a percentage of the colony counts from media lacking kanamycin. The recombinants were relatively stable after 7 generations as the percentage of colony forming units (cfu) that retained kanamycin resistance in the absence of ATc ranged from 86 - 100% (Table 3.4), however it dropped overtime and was <60 % after 42 generations.

Table 3.4: Retention of *rM. smegmatis*[TetRr1.12] plasmid stability in the absence of selection after multiple generations. The recombinants were grown without selection over 42 generations and the stability of the plasmids was then evaluated by colony count.

	Kan ⁻		Kan ⁺		Plasmid retention (%)
	Average colony counts from two experiments	Standard deviation (SD)	Average colony counts from two experiments	Standard deviation (SD)	
GENERATION 7					
pPM3r2.1	35	5.6	30	5.6	86
pPM3r2.2	29	2.8	26	4.2	90
pPM4r2.1	35	7.1	30	8.4	88
pPM4r2.2	23	5.7	23	4.2	100
GENERATION 42					
pPM3r2.1	58	4.2	28	2.8	48
pPM3r2.2	69	1.4	27	1.4	40
pPM4r2.1	53	2.8	26	4.2	59
pPM4r2.2	56	4.3	23	1.4	41

SD: Analysed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA, www.graphpad.com).

Plasmid retention %: Average colony count from Kan⁺ / Average colony count from Kan⁻

Overall, in the absence of kanamycin and ATc, plasmid instability occurred gradually. r*M. smegmatis* containing the *tetRr1.12* repressor gene in the same orientation as the *gfp* gene were more stable than the systems containing the gene in the opposite orientation.

3.3.3. ASSESSMENT OF THE TETRR1.12 REGULATORY SYSTEMS IN *M. bovis* BCG Δ PANCD

The TetRr1.12 plasmids were introduced into the pantothenic acid-deficient strain, *M. bovis* BCG mc² 6000 (*M. bovis* BCG Δ *panCD*). This auxotrophic strain was created by Bardarov *et al.*, (2002)²⁶⁷ using a specialized method of mycobacteriophage transduction. It contains deletions in the *panC* and *panD* genes that are involved in the *de novo* biosynthesis of pantothenate (vitamin B5), which is an essential requirement for lipid biosynthesis and metabolism contributing to the very complex and strong cell walls of mycobacteria. Consequently BCG Δ *panCD* requires supplementation with vitamin B5 for propagation²⁷¹.

Due to its auxotrophic nature, the growth of BCG Δ *panCD* is impaired in the phagosome environment where there is limited pantothenate, and this minimizes the risk of development of disseminated BCG⁵⁵. As a result the BCG Δ *panCD* strain is safer for use in immunocompromised hosts than the wild type BCG (Chapter 1.1), thus this strain was used for the generation of rBCG Δ *panCD* vaccine stocks in this study. Mayat, (2009)²⁶⁴ reported that the optimal concentration of vitamin B5 to supplement growth of BCG Δ *panCD* in order to approximate wild-type BCG growth was 24 μ g/ml. Furthermore, this strain has proven to be sensitive to ATc, making it an ideal strain for use in the expression of ATc controlled plasmids²⁶⁴.

3.3.3.1. Construction of the rBCG Δ *panCD*[TetRr1.12] systems

The TetRr1.12 plasmids were successfully transformed into BCG Δ *panCD* by electroporation followed by the preparation of rBCG Δ *panCD* vaccine stocks. Recombinant mycobacteria were then evaluated in terms of vector stability by restriction enzyme mapping of plasmids isolated from the vaccine stocks. Mapping verified the genetic integrity of all the rBCG Δ *panCD* plasmids.

3.3.3.2. Determination of the optimal concentration of anhydrotetracycline for rBCG $\Delta panCD$ [TetRr1.12] systems

The following experiment was performed to estimate the minimum ATc concentration that would inhibit GFP expression in rBCG $\Delta panCD$ [TetRr1.12] systems. Mayat, (2009)²⁶³ reported that the auxotrophic BCG $\Delta panCD$ is more resistant to ATc than *M. smegmatis*. In her study she demonstrated that the growth of this BCG $\Delta panCD$ is only significantly affected by ATc at 5 $\mu\text{g/ml}$. As a result a higher concentration range of ATc was used for the BCG $\Delta panCD$ as compared to the *M. smegmatis*. The recombinants were subjected to varying concentrations of ATc onto solid media in order to compare GFP fluorescence by visualization of the colonies (Figure 3.11).

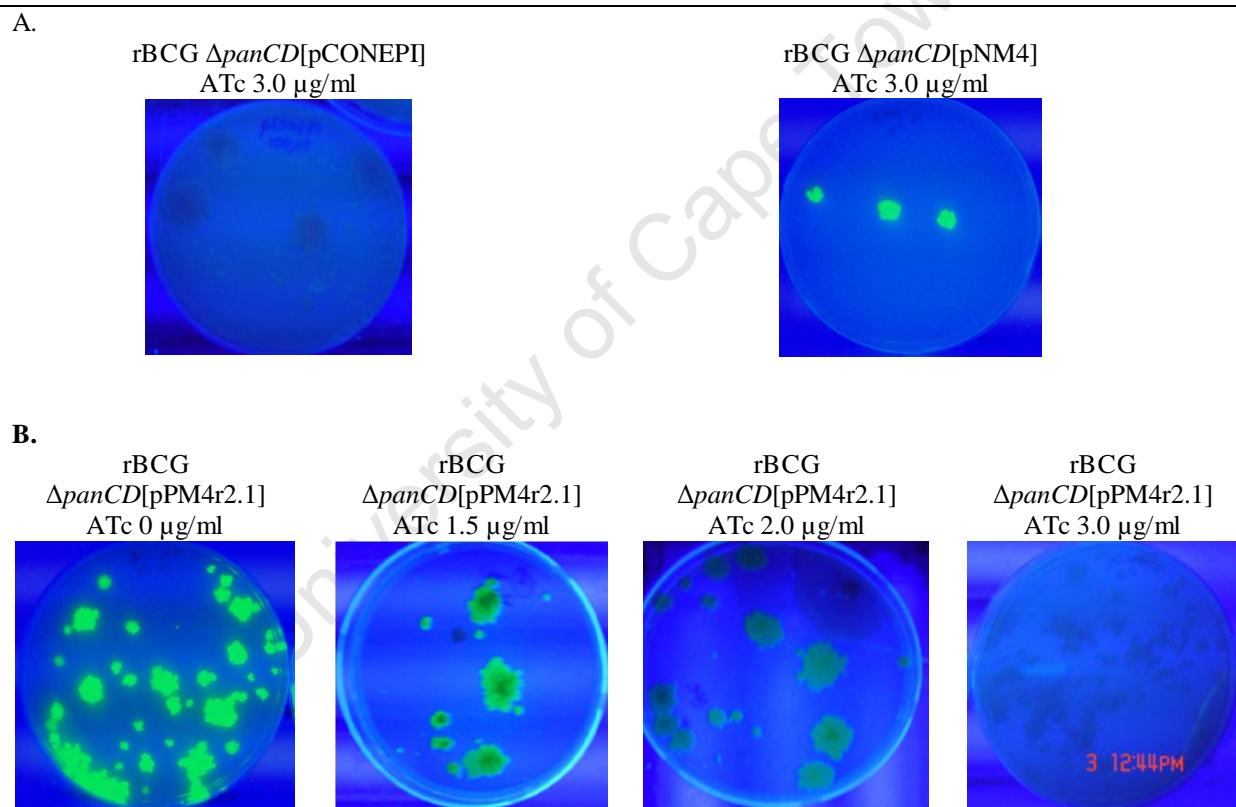


Figure 3.11: Images taken over UV light illustrating ATc-dependant repression of GFP expression of the rBCG $\Delta panCD$. A. controls, rBCG $\Delta panCD$ [pNM4] (positive) and rBCG $\Delta panCD$ [pCONEPI] (negative). B. Images showing the fluorescence of rBCG $\Delta panCD$ [pPM4r2.1] colonies on MB-7H10 medium containing various ATc concentrations.

All the recombinants displayed similar levels of ATc susceptibility, thus shown in the Figure 3.11 are the results obtained from one of the recombinants, rBCG $\Delta panCD$ [pPM4r2.1]. The different levels of fluorescence exhibited by the colonies on various amounts of ATc are depicted. As expected rBCG $\Delta panCD$ [pPM4r2.1] grown on ATc 0 $\mu\text{g/ml}$ displayed the highest levels of fluorescence. ATc at 1.5 $\mu\text{g/ml}$ did not completely repress GFP expression as the colonies showed a low level of fluorescence. ATc at 2.0 $\mu\text{g/ml}$ was selected as the optimal concentration seeing as it was the minimum concentration that repressed GFP expression. As expected the control rBCG $\Delta panCD$ colonies containing pNM4 and pCONEPI were not affected by the presence of ATc.

The next aim was to assess GFP expression in the presence and absence of ATc using a fluorimetry assay. Triplicate cultures of rBCG $\Delta panCD$ [TetRr1.12] were grown in liquid media, one was grown with 2.0 $\mu\text{g/ml}$ ATc, one with 3.0 $\mu\text{g/ml}$ ATc and the other without ATc. The cultures were then washed to remove ATc and GFP expression was induced for a period of 3-weeks. GFP expression was measured at weekly intervals, starting from week 0 (before the systems induced). The induction period for rBCG $\Delta panCD$ was lengthened compared to the period used for *M. smegmatis* considering BCG $\Delta panCD$ strain has a very slow growth rate.

Figure 3.12 shows the results obtained from one of the recombinants, rBCG $\Delta panCD$ [pPM4r2.1], at week 0 the GFP levels produced by rBCG $\Delta panCD$ [pPM4r2.1] grown with 2.0 $\mu\text{g/ml}$ ATc were very low (approximately 2-fold) compared to the system grown without ATc, which indicated good repression. The levels of GFP gradually increased from 1 to 3 weeks post ATc removal and the highest level was obtained after 3 weeks, which were approximately 5.7-fold higher than the levels produced prior to ATc removal. rBCG $\Delta panCD$ [pPM4r2.1] grown with 3.0 $\mu\text{g/ml}$ ATc showed better repression since the GFP level was >2-fold lower than the system grown without ATc, however the system induced very slow after ATc was removed. rBCG $\Delta panCD$ [pPM4r2.1] grown in the absence of ATc produced the highest level of GFP after 2 weeks and then started dropping afterwards, which was an indication of plasmid instability.

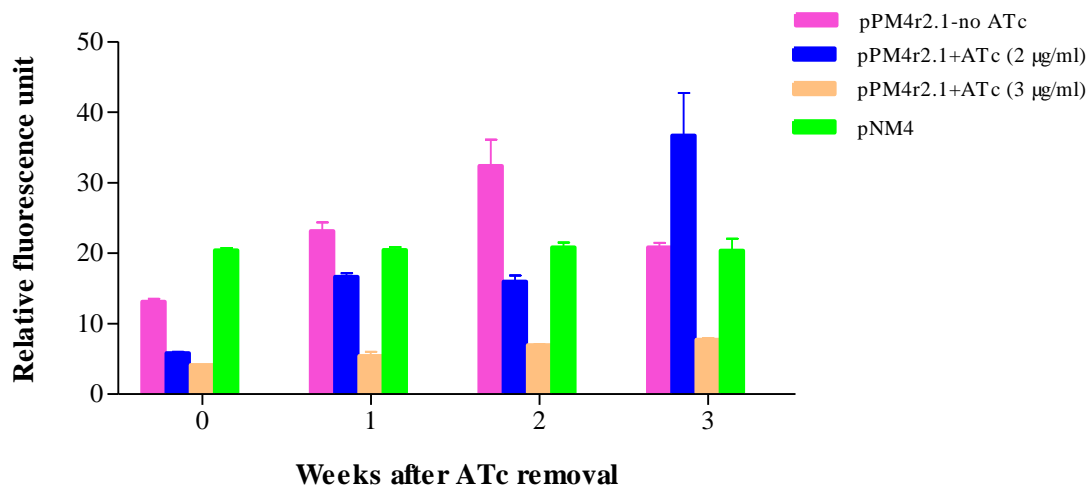
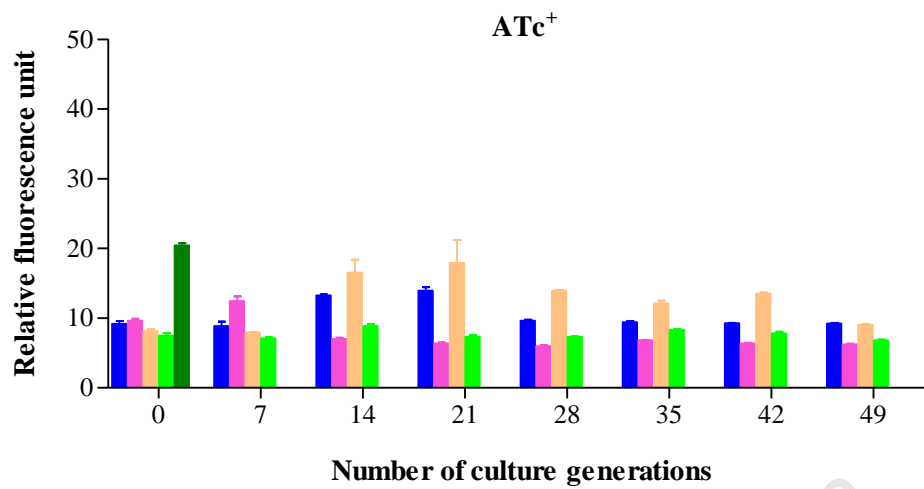


Figure 3.12: Assessment of repression and induction of GFP expression in rBCG $\Delta panCD$ [pPM4r2.1]. GFP expression was measured by fluorimetry assay and expressed as relative fluorescence units after subtracting the background readings of rBCG $\Delta panCD$ [pCONEPI]. The positive control was rBCG $\Delta panCD$ [pNM4]. Error bars represent the standard deviations from two experiments.

3.3.3.3. Assessment of genetic stability of rBCG $\Delta panCD$ [TetRr1.12]

As previously mentioned, genetic instability is one of the major factors impeding the commercial production of rBCG-based vaccines (Chapter 1). This experiment was designed to assess the genetic integrity of the rBCG $\Delta panCD$ vaccine vectors expressing GFP after 49 generations. Similar to *M. smegmatis* systems, the genetic stability of the rBCG $\Delta panCD$ [TetRr1.12] was evaluated with and without ATc over many generations, in order to determine the maintenance of the *gfp* gene, retention of the Kan^R gene and the percentage of plasmid loss. The number of passages was increased to seven (\approx 49 generations) in order to lengthen the rBCG $\Delta panCD$ vaccine vector assessment period as stability impacts on the preparation of the vaccines on a large scale. In addition, the colony morphology was assessed in order to examine the degree at which metabolic load was exerted on the BCG strain since the size of colonies reflects their growth rate. GFP expressed by the recombinants was monitored by fluorimetry assay. As seen in Figure 3.13, the presence of ATc reduced the levels of GFP (panel A) compared to the levels of GFP produced in the absence of ATc (panel B). GFP expression in the recombinants grown with ATc appeared to be low and consistent for 49 generation, whereas GFP expression in the recombinants grown without ATc showed steady reduction overtime.

A.



B.

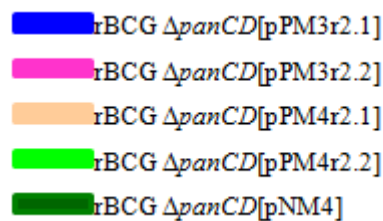
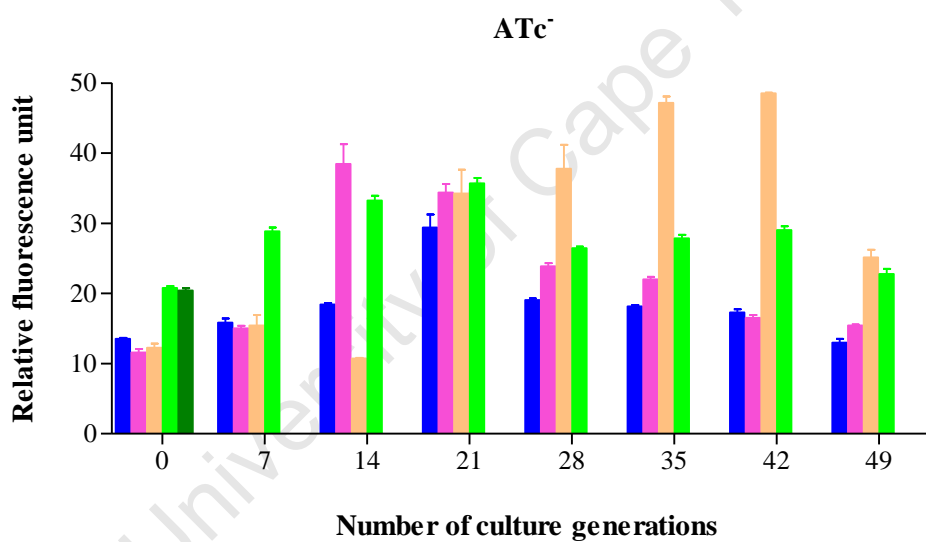


Figure 3.13: Assessing the stability of the rBCG $\Delta panCD$ [TetRr1.12]. GFP expression in the systems was assayed in the presence and absence of ATc using fluorimetry and expressed as relative fluorescence units after subtracting the background readings of rBCG $\Delta panCD$ [pCONEPI]. Fluorimetry readings of the systems in, **A.** ATc⁺ and **B.** ATc⁻. rBCG $\Delta panCD$ [pNM4] was the positive control. Error bars represent the standard deviations from three experiments.

In our laboratory it has been demonstrated that reduction in the growth rate of the host cells can be linked to an over-expression of recombinant antigens and a build up of metabolic load, where the recombinants produce small or unhealthy colonies^{263, 270}. In this experiment a comparison of colony morphology between colonies produced after 7 generations and those produced after 49 generations was done by visualization of the recombinant colony fluorescence over a UV trans-illuminator.

Figure 3.14 depicts rBCG *ΔpanCD* colonies after 7 generations. Although the colonies displayed slightly lower levels of fluorescence in the presence of 2.0 μg/ml ATc than in its absence, the magnitude of repression was not as intense as observed in the liquid media at the same concentration of ATc. Moreover, the recombinants produced comparable colonies in the presence and absence of ATc, in terms of the size. Figure 3.15 show rBCG *ΔpanCD* colonies produced after 49 generations. In the presence of ATc, the colonies were very similar to those produced after 7 generations, in terms of fluorescence intensity and size. Colony sizes were not uniform, either in the presence or absence of ATc. However, in the absence of ATc colonies showed a general reduction in size and more intense fluorescence. In our experience both transformed and untransformed BCG colonies are fairly heterogenous in size. This data indicates that the colony sizes were inversely correlated to GFP expression levels.

Plasmid stability of the rBCG *ΔpanCD* was assessed by counting the colonies produced by the recombinants after passaging over 49 generations in the absence of selection as performed in r*M. smegmatis*. The recombinants were relatively stable after 7 generations as the percentage of cfu that retained antibiotic resistance in the absence of kanamycin ranged from 82 - 91%, however it dropped overtime as instability increased and ended up between 63 and 73% after 49 generations (Table 3.5).

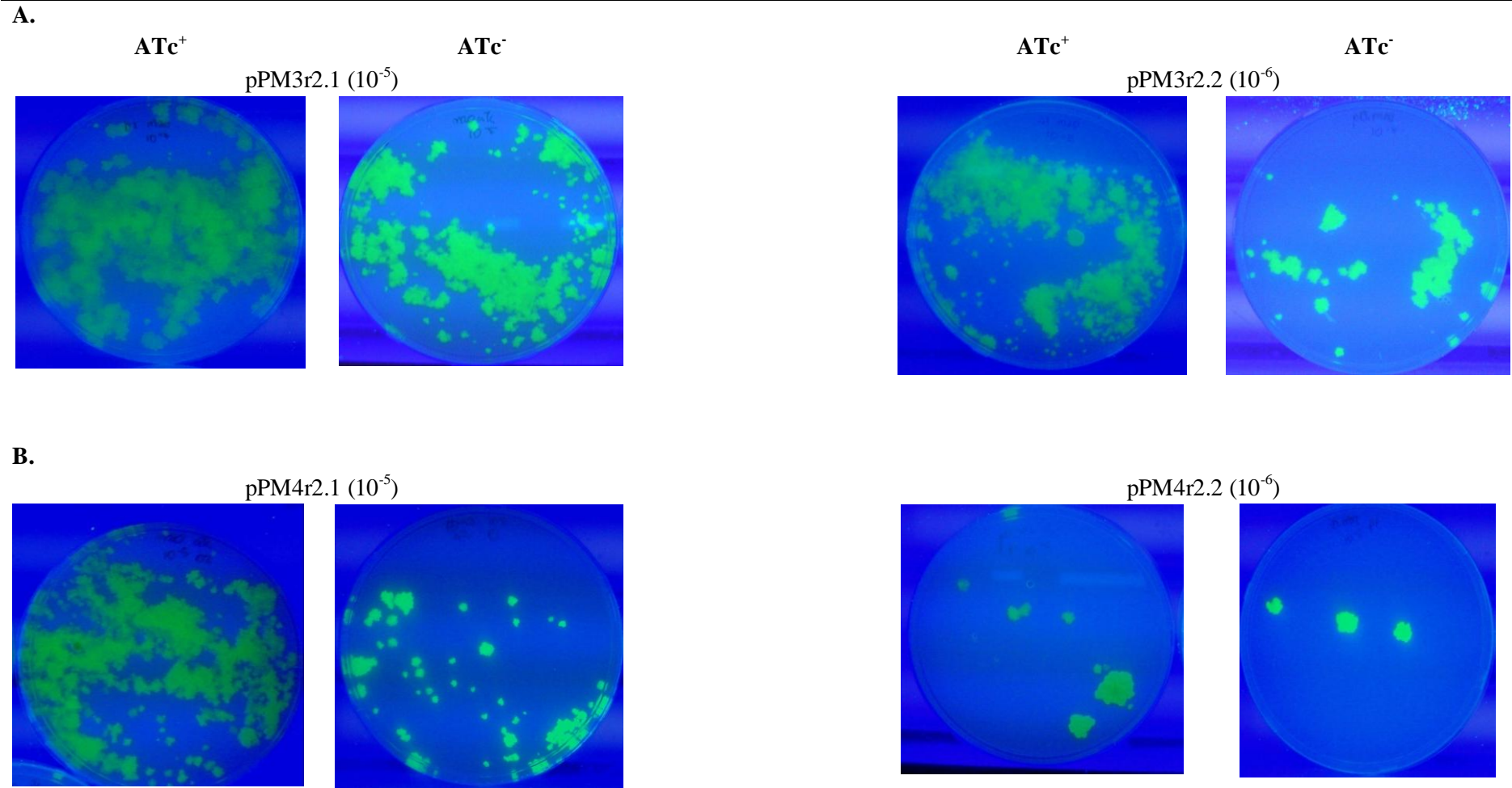


Figure 3.14: Images of the rBCG *ΔpanCD*[TetRr1.12] colonies taken over UV light after 7 generations. Colonies produced after culturing the recombinants over 7 generations in the presence and absence of anhydrotetracycline.

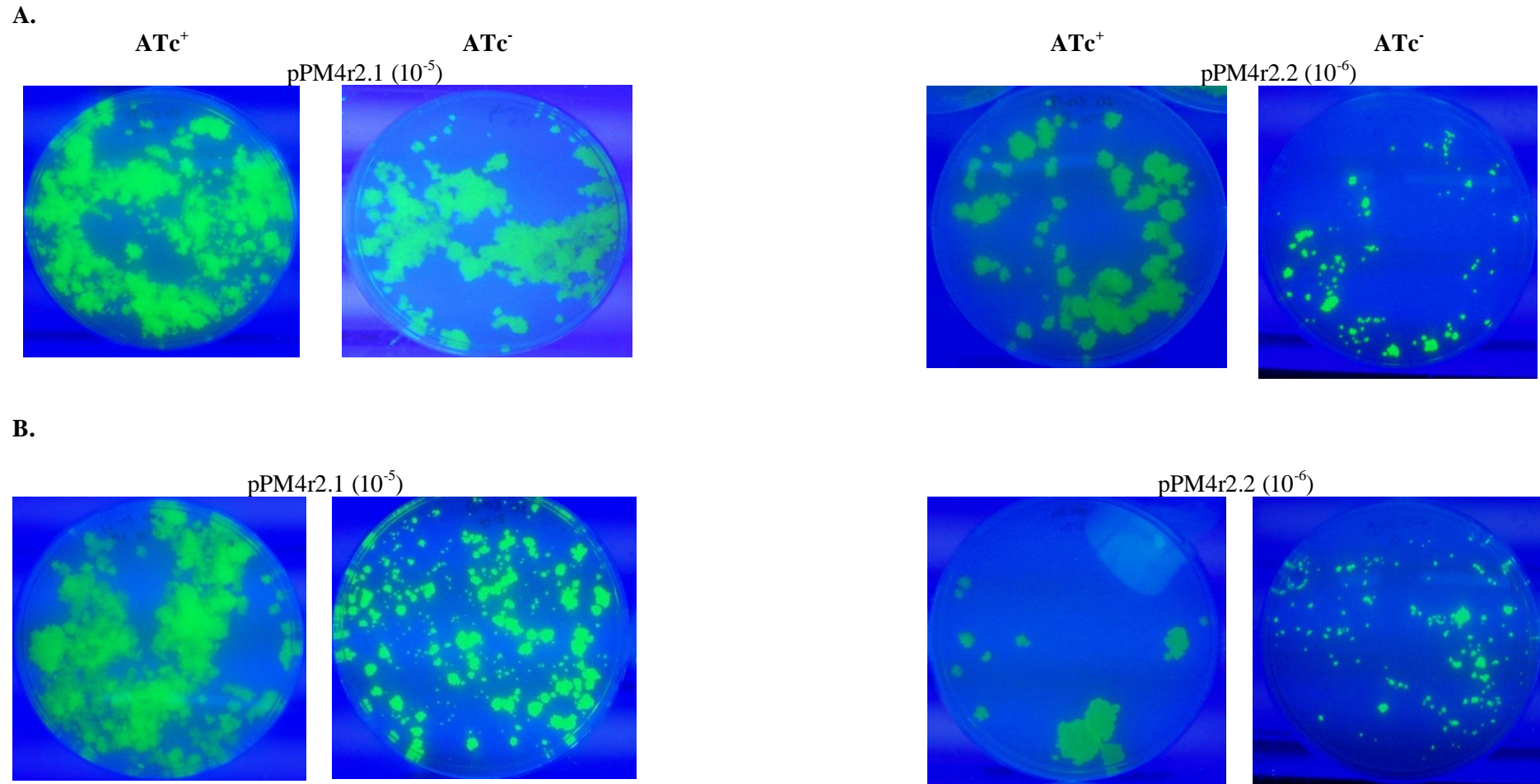


Figure 3.15: Images of the rBCG *ΔpanCD*[TetRr1.12] colonies taken over UV light after 49 generations. Colonies produced after culturing the recombinants over 49 generations in the presence and absence of anhydrotetracycline.

Plasmid stability was also evaluated by restriction enzyme mapping of plasmid DNA isolated from the rBCG $\Delta panCD$ [TetRr1.12] cultures produced after 49 generations in presence and absence of ATc. A total of 20 clones were mapped for recombinant (data not shown), and then the percentages of the stable clones were determined. In the presence of ATc the plasmids remained genetically stable since 100% of the recombinants tested produced expected band patterns, whereas in the absence of ATc the percentage of stability varied between the recombinants, rBCG $\Delta panCD$ [pPM3r2.1] displayed 76% stability, rBCG $\Delta panCD$ [pPM3r2.2] displayed 74% stability, rBCG $\Delta panCD$ [pPM4r2.1] displayed 81% stability and rBCG $\Delta panCD$ [pPM4r2.2] displayed 57% stability.

Overall, the TetRr1.12 vectors were more genetically stable in BCG $\Delta panCD$ than in *M. smegmatis*. Furthermore, TetRr1.12 vectors containing the *tetRr1.12* repressor gene in the same orientation as the *gfp* gene (pPM3r2.1 and pPM4r2.1) were more stable than the systems containing the gene in the opposite orientation (pPM3r2.2 and pPM4r2.2) in both mycobacterial strains.

Table 3.5: Assessment of rBCG $\Delta panCD$ [TetRr1.12] plasmid stability retention in the absence of selection after multiple generations.

	Kan⁻		Kan⁺		Plasmid retention (%)
	Average colony counts from two experiments	Standard deviation (SD)	Average colony counts from two experiments	Standard deviation (SD)	
GENERATION 7					
pPM3r2.1	28	9.8	23	4.2	82
pPM3r2.2	24	7.1	22	7.1	91
pPM4r2.1	23	2.1	19	5.6	82
pPM4r2.2	20	8.4	17	7.1	85
GENERATION 49					
pPM3r2.1	23	14.1	17	2.8	73
pPM3r2.2	20	17.0	13	1.4	65
pPM4r2.1	21	4.2	15	15.6	71
pPM4r2.2	19	7.1	12	1.4	63

The recombinants were grown without selection for \pm 49 generations and the stability of the plasmids were then evaluated by colony count as shown below.

SD: Analysed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA, www.graphpad.com).

Plasmid retention %: Average colony count from Kan⁺/ Average colony count from Kan⁻

3.4. DISCUSSION

In this study the aim was to express a recombinant antigen in an episomal vector under a strong promoter to ensure sufficient levels were produced. However we wanted to avoid vector instability, therefore an expression system was constructed, where a recombinant antigen was placed under a regulatable promoter. In the system the regulation of transcription of the recombinant antigen was facilitated by switching the promoter controlling it on and off with the aid of a TetRr1.12 repressor and a chemical co-repressor, ATc.

Using the TetRr1.7 repressor Guo *et al.*, (2007)¹⁹⁷ observed that the silencing of the recombinant antigen was only achieved if the repressor was expressed by a strong promoter. From their data we can deduce that the regulation of the system is not only influenced by the concentration of the co-repressor but also by the quantity of the repressor. Therefore, to ensure efficient regulation of the system, the *tetRr1.12* gene was placed under the control of a strong promoter (*hsp60*) in order to ensure that sufficient levels of the repressor were produced.

A series of the episomal TetRr1.12 regulatory systems, pPM3r2.1, pPM3r2.2, pPM4r2.1 and pPM4r2.2 were constructed. The systems consisted of different combinations of *tetO* operators (in the $P_{m_{cyc}}$ promoter region) and the TetRr1.12 expression cassette (*tetRr1.12* gene expressed from the *hsp60* promoter) in different orientations. These were evaluated using GFP as a model antigen, where the expression was monitored by measuring the intensity of fluorescence using a fluorimetry assay. In the proof of concept experiment the systems responded to ATc by showing repression of GFP expression in its presence. The systems containing the *tetRr1.12* gene in the opposite orientation to the *gfp* gene, pPM3r2.2 and pPM4r2.2 were more sensitive to ATc, whereas the systems containing the *tetRr1.12* gene in the same orientation as the *gfp* gene, pPM3r2.1 and pPM4r2.1 were less sensitive to ATc. This indicated the existence of a correlation between the orientation of the *tetRr1.12* gene and ATc sensitivity. We suspect that the DNA conformation (the *tetRr1.12* gene orientation) around the $P_{m_{cyc}}$ promoter region may influence the binding of the TetRr1.12-ATc complex to the *tetO* operator sequences.

Next, the optimal concentration of ATc was determined, i.e. the minimum ATc concentration that would repress GFP expression and could be removed without difficulty to allow induction of recombinant antigen expression. In *M. smegmatis* the optimal ATc concentration for the systems ranged from 100 - 250 ng/ml (Figure 3.7), which lies within the published ATc range of 10 - 300

ng/ml that has been used for an efficient silencing of genes in *M. smegmatis* with the TetRr1.7 repressor^{197, 198}. In *M. smegmatis* up to 2.5-fold repression at a dose of 100 ng/ml ATc was seen compared to r*M. smegmatis* grown in the absence of ATc. In BCG $\Delta panCD$, although 2.0 μ g/ml ATc did not achieve complete repression (Figure 3.11 & 3.12), it was selected as the optimal ATc concentration for the TetRr1.12 systems since the maximal induction of GFP expression following the removal of ATc was not achieved when the cultures were grown in media containing higher concentration of ATc. Also a very low level of recombinant antigen expression was not expected to cause genetic instability. Repression achieved with 2.0 μ g/ml ATc was approximately 2-fold in all the systems. In comparison to the codon optimized TetRr1.7 repressor, which mediated a 3-fold repression in the presence of 100 ng/ml ATc in *M. smegmatis*²⁶⁴, the codon optimized *tetRr1.12* was seemingly less efficient as it required a higher concentration of ATc to repress GFP. This data is in accordance with the published data as Scholz *et al.*, (2004)²⁵⁴ reported that the TetRr1.7 repressor is more efficient than the TetRr1.12 repressor. Optimal ATc concentrations were favourably lower than the MIC of both of mycobacterial strains, which are 400 ng/ml for *M. smegmatis* and ~5000 ng/ml for BCG $\Delta panCD$ ²⁶⁴.

The induction kinetics data showed slow induction of the r*M. smegmatis* cultures as none of these were maximally induced 72 hrs post ATc removal (Figures 3.8 and 3.9). Inefficient induction may have been caused by elevated levels of the TetRr1.12 repressor as it was expressed from a replicative plasmid and placed under a strong promoter, which might have obstructed the initiation of the P_{smyc} promoter. According to Lutz and Bujard, (1997)¹⁹³ if over-expressed, the repressor can interfere with the induction of the promoter activity by strongly binding to the *tetO* operators. On the other hand, rBCG $\Delta panCD$ showed complete induction 3 weeks after ATc removal and no indication of genetic instability (Figure 3.12).

This study also showed that the induction of the P_{smyc} promoter after removal of ATc is time- and dose-dependent as the induction kinetics positively correlated with time and the expression levels of the recombinant antigen varied in response to the ATc doses. The recombinants cultured in the presence of lower ATc concentrations induced faster and achieved the highest GFP levels after ATc removal, whereas the recombinants cultured in the presence of higher concentrations of ATc induced at a lower rate and produced lower levels of GFP. This may be because TetR1.12 repressor protein-ATc complex binds the operator sequences fairly strongly

and irreversibly. Thus at higher concentrations of ATc almost all the operator sequences are bound inhibiting expression of GFP. This was consistent with the data generated by Mayat (2009)²⁶⁴ (reviewed in section 3.1). Also a trend was observed in *M. smegmatis*, in which there was a correlation between the GFP levels and growth phase in the presence and absence of ATc (Figures 3.8 and 3.9). The increase in GFP expression during stationary phase growth (12 - 24 hrs) may have resulted from variations in P_{smyc} promoter activity. We assume that P_{smyc} promoter expression might be highest during late stationary growth phase.

There were no significant differences between the systems harbouring the pNM3 (*tetO*₂ operator sequences) and those harbouring the pNM4 (*tetO*₁ operator sequences) in both mycobacterial strains. This was unexpected as the affinities of the TetRr1.12 repressor for the *tetO*₁ and *tetO*₂ operators are not identical^{197, 226}. We expected the ATc-TetRr1.12 complexes to bind more strongly to the *tetO*₂ and inhibit the expression more intensely, i.e the pNM3 systems to be more repressed than the pNM4 systems, and the ATc-TetRr1.12 complexes to dissociate from the *tetO*₁ faster and more efficiently, i.e the pNM4 systems to induce faster than the pNM3 systems after ATc removal. However, our results were in agreement with the results reported by Mayat (2009)²⁶⁴ when she compared the promoter-operator designs in her multicopy episomal system (containing the *tetRr1.7* gene). She reported that the levels of GFP expression in the pNM3 and pNM4 systems were comparable.

The ability of the TetRr1.12 systems to maintain vector genetic stability over a number of culture generations in the presence of ATc, and post ATc removal should allow preparation of stable vaccines on a large scale and ensure that the vaccines will be intact upon vaccination of the animal models and produce sufficient antigen to induce a good immune response. As reviewed in Chapter 1, over-expression of a recombinant antigen results in a metabolic load within the host cell, which leads to genetic instability of the vaccine vector and a decrease in the growth rate of the recombinants. On solid media increased metabolic load is indicated by the reduction in relative colony size^{167, 272}. Genetic stability assessment was analysed after passaging the recombinants for many generations (42 generations in *M. smegmatis* and 49 generations in BCG *ΔpanCD*) with regulation (ATc⁺) and without regulation (ATc⁻). In the absence of ATc, *rM. smegmatis* became genetically unstable, which was depicted by the decrease in the number of fluorescing colonies overtime. Plasmid instability was observed following restriction enzyme mapping as <56% of plasmids isolated from recombinants harbouring the *tetRr1.12* gene in the

same orientation as the *gfp* gene and <2% harbouring the *tetRr1.12* gene in the opposite orientation to the *gfp* gene were genetically intact (Figure 3.10). Instability occurred as early as after the 7th culture generation. In BCG $\Delta panCD$, the recombinant colonies produced after 7 generations were comparable to those produced in its presence in terms of size (Figure 3.14), indicating that the induced systems were still genetically intact and the metabolic load had not built up. However after 49 generations most of the colonies were fluorescing strongly and were smaller in size (Figure 3.15), which was an indication of GFP over-expression and thus evidence of metabolic load. Plasmid mapping revealed that instability occurred after the 35th culture generation in all rBCG $\Delta panCD$ systems, 76 - 81% recombinants harbouring the *tetRr1.12* gene in the same orientation as the *gfp* gene and 57 - 74% recombinants harbouring the *tetRr1.12* gene in the opposite orientation to the *gfp* gene remained genetically intact. Without regulation instability was expected as our systems were vectored by an episomal vector and the recombinant antigen was expressed from a strong promoter. Several studies have demonstrated *in vitro* instability displayed by episomal-based rBCG vaccines expressing HIV and SIV antigens (Reviewed in Chapter 1). Mederle *et al.*, (2002)⁹⁵ used the pAL5000-derived replicative and the Ms6-derived integrative vectors to generate a rBCG expressing SIV antigens. Although the integrative rBCG expressed lower levels of the antigens, it was genetically more stable both *in vitro* and *in vivo*, whereas the episomal rBCG displayed low genetic stability and rapidly lost the plasmid *in vivo*.

The presence of ATc on the other hand lessened genetic instability. In *M. smegmatis* there was no drastic decrease in the number of fluorescing colonies after 42 generations (Figure 3.10), and no colony size reduction was observed. No genetic instability was observed following restriction enzyme mapping of plasmid DNA isolated from *rM. smegmatis*. In BCG $\Delta panCD$ the presence of ATc reduced the expression of GFP as signified by low fluorescence intensity, which reduced metabolic load and as a result recombinant colonies appeared large and healthy even after several generations (Figure 3.15). Furthermore, the rBCG $\Delta panCD$ maintained genetic stability for 49 generations as DNA mapping of the plasmids confirmed 100% genetic stability.

In the repression study of rBCG $\Delta panCD$ the results obtained in liquid medium did not completely correlate with those obtained on solid medium, i.e. the colonies (solid medium) fluoresced brightly in the presence of 2.0 $\mu\text{g/ml}$ ATc (Figure 3.13), whereas low fluorimetry readings (liquid medium) were observed in the presence of the same amount of ATc (Figure

3.14). Three rationales could explain the differences in results obtained from these two assays. The first could be that the amount of active ATc molecules available to repress the recombinants were constantly high in liquid media since ATc was added freshly when cultures were passaged, whereas on solid media ATc was only added once and the plates were incubated for 4 weeks during which time the ATc might have become inactive. There do not seem to be any publications on the half-life of ATc in mycobacteria, however it is 6 hours in humans (37°C) ²⁷³. The second could be the dilution of ATc molecules as several numbers of generations of bacterial growth occur to form each colony and the rate of diffusion of ATc in solid media may be slower than in liquid media, which could result in a lower concentration of ATc around mycobacterial colonies on plates as compared to in cultures. The third could be that the fluorimetry assay is a more sensitive method than the visualization over UV light.

Overall, our data showed different trends displayed by the TetRr1.12 systems in terms of foreign gene expression and genetic instability in *M. smegmatis* and BCG $\Delta panCD$. We showed that in the absence of ATc, the recombinants over-expressed GFP, which increased toxicity or the metabolic load on the cell and in turn slowed down the growth rate. These results were in agreement with those reported by Mayat (2009)²⁶⁴ when she compared the levels of GFP expression and colony morphology displayed by BCG $\Delta panCD$ and Pasteur transformants harbouring different plasmids. She reported that colony sizes were inversely correlated to GFP expression levels, such that the high level of GFP expression inflicted metabolic load on the bacteria as the smaller colonies fluoresced very highly. Despite incomplete repression of GFP, the presence of the ATc reduced the metabolic load on the bacteria, which improved genetic stability. Plasmids, pPM3r2.1 and pPM4r2.1 were the most stable TetRr1.12 systems in both strains. Furthermore, the rBCG $\Delta panCD$ systems were genetically more stable and displayed better induction capacity than the *rM. smegmatis* after removal of ATc, which was pleasing since this strain was to be used for generation of the vaccines expressing HIV antigens.

CHAPTER 4: CONSTRUCTION AND EVALUATION OF THE EPISOMAL MULTICOPY HIV-T_{ET}R SYSTEMS

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4.1. INTRODUCTION

Sub-Saharan Africa is greatly affected by the progressive HIV endemic, where millions of people die annually from AIDS²⁷⁴⁻²⁷⁶. There is an urgent need for a prophylactic HIV vaccine that would help prevent new infections and reduce the virus in the infected individuals. rBCG expressing HIV antigens have shown potential as good HIV vaccine candidates due to their persistent replication following immunization that enable them to continually stimulate the immune system (reviewed in Chapter 1). Most rBCG vaccines developed for HIV aim to elicit long-lasting antibody-mediated and T-cell-mediated immune responses in both the mucosal and systemic components of the immune system^{106, 134}. These immune responses are able to control chronic disease and thus may be able to control HIV viral load and limit progression to AIDS given that they target both cell-free and associated modes of HIV infection¹⁴³⁻¹⁴⁶.

BCG-based vectors have shown promise as an HIV vaccine vector since the early 1990s, however the circuitously linked phenomena, genetic instability and low heterologous antigen expression have impeded their use in clinical trials (reviewed in Chapter 1). These negative characteristics are thought to result in mutation of the gene of interest or the loss of the entire plasmid from the bacterium resulting in low or no heterologous antigen production.

In the previous chapter a comprehensive description and evaluation of the construction of various designs of the ATc-dependent TetRr1.12 regulatory systems was given. The TetRr1.12 regulatory systems contain the regulatory and the responsive components of the TetR system for stringent regulation of recombinant antigen expression, i.e. a revTetR cassette; and a strong P_{smyc} mycobacterial promoter engineered to contain *tetO* operator sequences. These components efficiently facilitated the regulation of the expression of GFP in *M. smegmatis* and BCG $\Delta panCD$ strains. We evaluated the genetic stability and the expression capacity of the TetRr1.12 systems in *M. smegmatis* and BCG $\Delta panCD$. Our data showed that the TetRr1.12 systems, pPM3r2.1 and pPM4r2.1 containing the *tetRr1.12* gene in the same orientation as the *gfp* gene were the most stable in both *M. smegmatis* and BCG $\Delta panCD$ strains. Thus these systems were selected to incorporate the HIV-1 antigens to generate the episomal multicopy HIV-TetRr1.12 systems.

In this chapter we were aiming to develop a stable rBCG $\Delta panCD$ vaccine expressing HIV antigens from the HIV-1 subtype C virus. We used HIV-1 subtype C, *rt* and *gag* genes because the HIV-1 subtype C virus is predominant in southern Africa and is also responsible for the majority of the HIV infections globally ²⁷⁷⁻²⁸⁰.

The HIV *gag* gene encodes the HIV-1 Gag polyprotein precursor (Pr55^{gag}) that includes the matrix protein (p17), the capsid protein (p24), a spacer protein (p2), the nucleocapsid protein (p7), spacer protein (p1) and p6 (plays a role in the release of mature HIV virions from the cell) ^{281, 282}. Gag is the most commonly used HIV antigen in HIV vaccine studies due to its conservative nature across the HIV-1 subtypes ²⁸¹. The HIV *rt* gene is a part of the *pol* gene. Not only is the *pol* gene well conserved across the HIV-1 subtypes, but its products also contain immunodominant epitopes ^{283, 284}. The *rt* gene encodes the enzyme, reverse transcriptase, an RNA-dependent polymerase that is important for viral replication as it transcribes a single stranded RNA from the virion into a double-stranded DNA proviral DNA ²⁸². This enzyme is error-prone and thus increases the mutation rate of HIV, which facilitates the virus's immune escape.

It was recently reported that HIV Pol and Gag-specific responses are essential for effective cellular immunity ^{130, 146, 285}, as studies have demonstrated that the strongest effective CTL specific responses are directed towards these antigens ^{283, 286, 287}. Ramduth *et al.*, (2005)²⁸⁸ demonstrated that Gag-specific responses are the dominant CD4⁺ T-cell response to HIV and that the CD8⁺ T-cells to Gag are associated with lower viraemia. Consequently we utilised the HIV-1 genes, *rt* and *gag* in our study to construct a candidate vaccine aimed at stimulating good and effective CTL responses.

4.1.1 STUDY OBJECTIVES

The objectives were to:

- i) generate the HIV-TetRr1.12 systems by inserting HIV-1 subtype C, *rt* and *gag* genes into the stable ATc-dependent TetRr1.12 regulatory systems
- ii) introduce the systems into the BCG $\Delta panCD$ strain
- iii) expand the recombinants *in vitro* in the presence of ATc in order to down-regulate the expression of the HIV-1 antigens
- iv) remove ATc to induce the HIV-1 antigen expression
- v) prepare rBCG $\Delta panCD$ vaccines stocks at the time point, where the highest level of heterologous antigen expression is achieved
- vi) compare the rBCG $\Delta panCD$ that utilise the TetRr1.12 repressor for regulation to the recombinants that utilise the TetRr1.7 repressor, in terms of HIV antigen expression genetic stability and immunogenicity.

The experimental plan is depicted graphically in Figure 4.1.

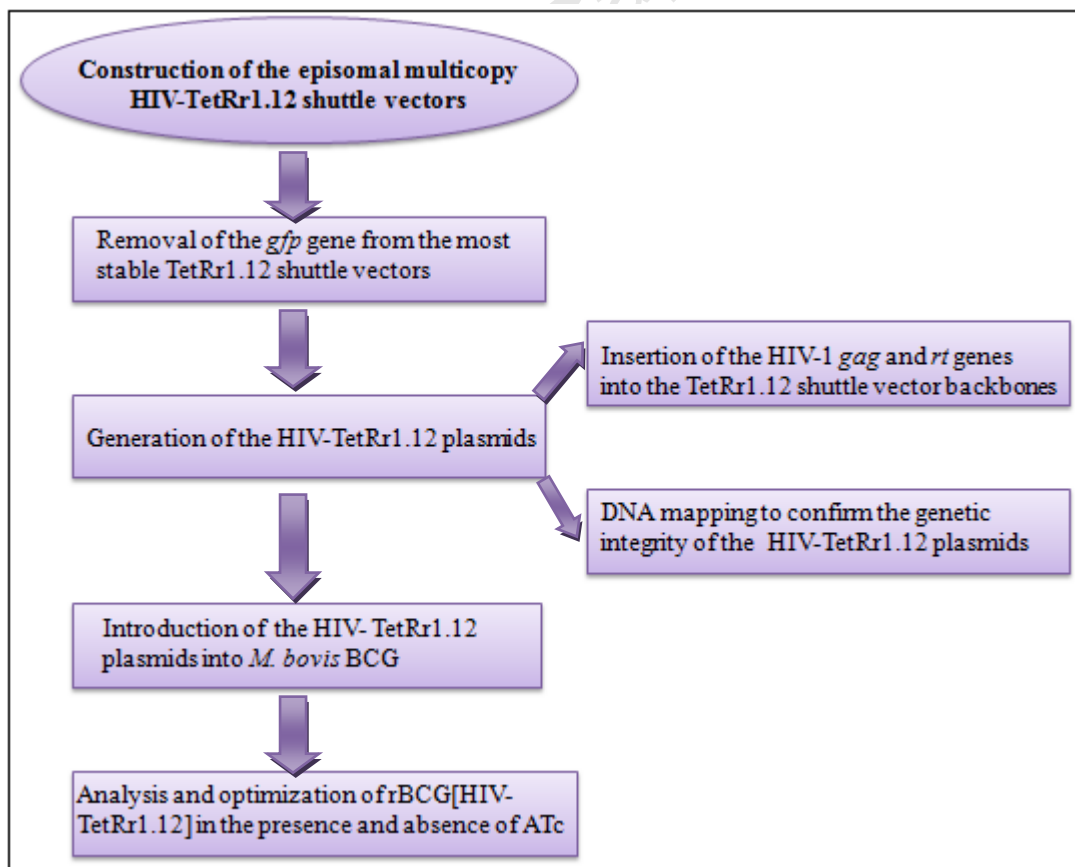


Figure 4.1: A schematic illustration of the project plan for this Chapter.

4.2. MATERIALS AND METHODS

4.2.1. GENERATION OF THE HIV-TetRr1.12 SHUTTLE VECTORS

4.2.1.1. Insertion of the HIV-1 antigen genes into the TetRr1.12 plasmids

Mayat, (2009)²⁶⁴ observed that the *rM. smegmatis* containing *tetRr1.7* in the opposite direction to the *gfp* gene were less stable than those containing *tetRr1.7* in the same direction as the *gfp* gene. She concluded that *rM. smegmatis*[pNM3.1] was the most stable system. Consequently this plasmid was also selected to incorporate the HIV-1 genes in order to generate the HIV-TetRr1.7 systems. The HIV-TetRr1.7 systems were constructed by Dr Ros Chapman in our laboratory, whereby she removed the *gfp* gene from the plasmid pNM3.1 and replaced it with the HIV-1 antigens to generate the plasmids pRC3Gag and pRC3RT (Appendix E10 & 11).

TetRr1.12 plasmids, pPM3r2.1 and pPM4r2.1 (construction described in the chapter 3, maps shown in Appendix E6 & 8) were used to incorporate the HIV-1 antigen genes as shown in Figures 4.2 & 4.3. A double digest with restriction enzymes *EcoRI* + *HpaI* [Fermentas] that flank the *gfp* gene was used to excise it from the TetRr1.12 plasmids. The HIV-1 subtype C *gag* and *rt* genes were excised from the plasmids pRC3Gag and pRC3RT respectively, using restriction enzymes *EcoRI* + *HpaI*. The HIV-1 genes are linked to the V3-SV5 epitope, a 10 amino acid V3 CTL tag and an SV5 monoclonal antibody tag. All the DNA fragments were gel purified using QIAquick Gel Extraction Kit [Qiagen] according to the manufacturer's instructions and then ligated according to Appendix B4.

4.2.1.2. Confirmation of the positive HIV-TetRr1.12 plasmids by plasmid DNA mapping

Ligation mixes (5 µl) were transformed into *E. coli* (Appendix A3), cultured and the small-scale plasmid DNA was isolated from the cultures (Appendix B1.1). To confirm the presence of the HIV-1 *rt* and *gag* genes restriction enzyme mapping of plasmid DNA was performed using a double *HpaI* + *XbaI* and a single *EcoRV* restriction enzyme digest, respectively (Appendix B2). The confirmed HIV-TetRr1.12 plasmids were subjected to a large-scale plasmid isolation procedure (Appendix B1.2).

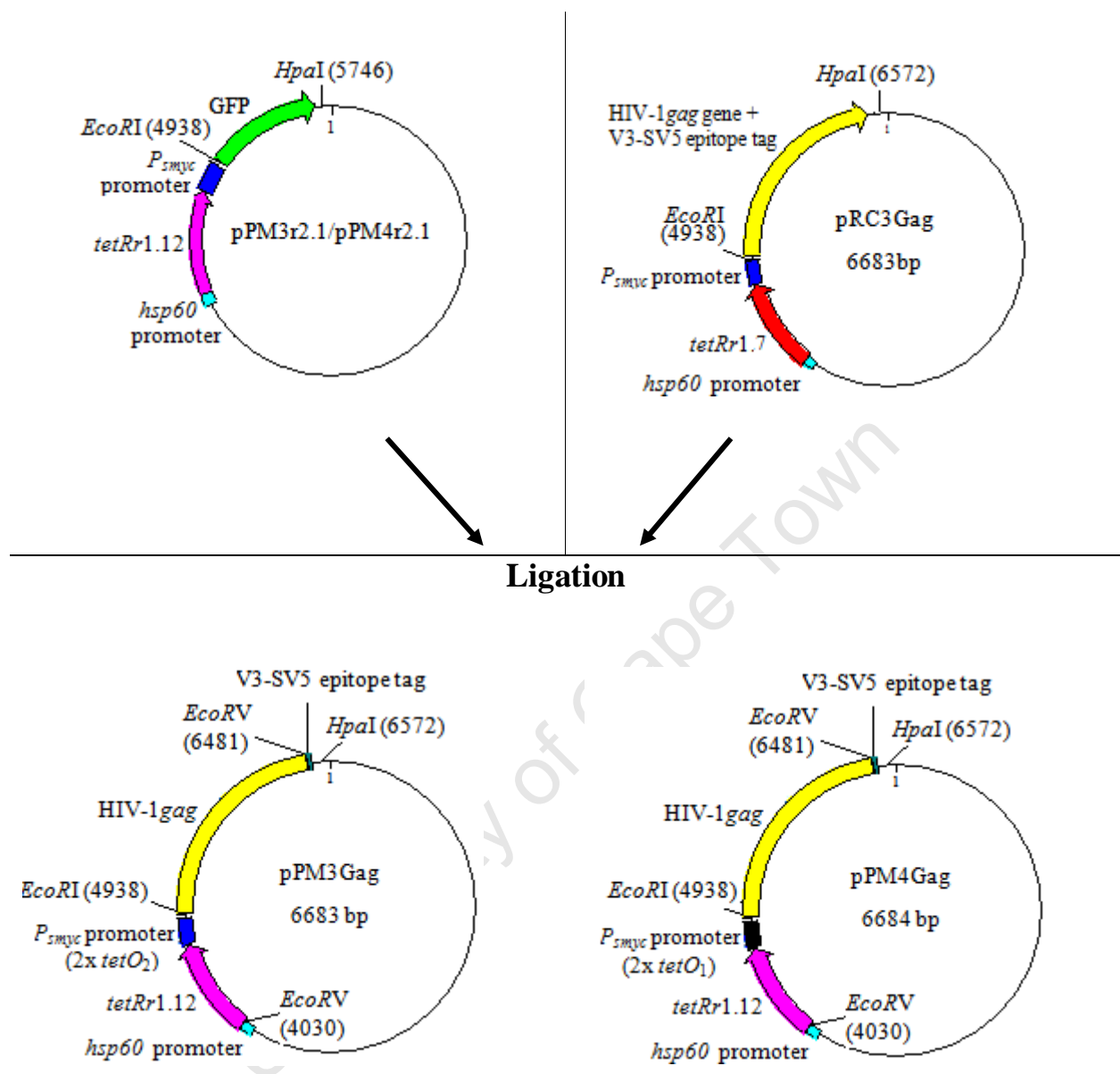


Figure 4.2: Illustration of the generation of the HIV-1 Gag-TetRr1.12 plasmids. The TetRr1.12 plasmids containing the TetRr1.12 cassette in the same orientation as the GFP, pPM3r2.1 (P_{smyc} promoter with $2x tetO_2$) and pPM4r2.1 (P_{smyc} promoter with $2x tetO_1$) were linearized and ligated with the HIV-1gag + V3-SV5 epitope tag from plasmid pRC3Gag to generate plasmids, pPM3Gag and pPM4Gag.

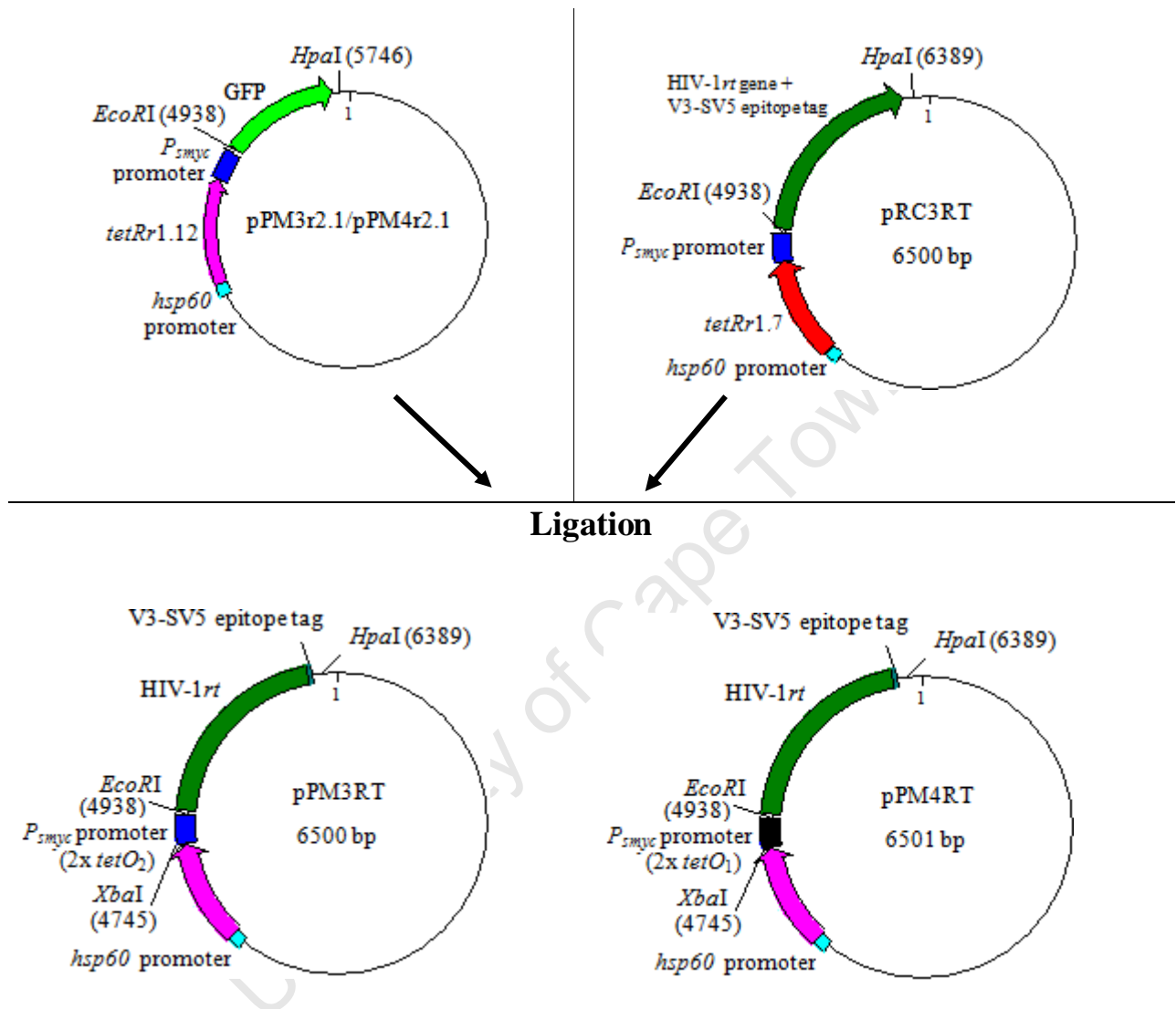


Figure 4.3: Illustration of the generation of the HIV-1 RT-TetRr1.12 plasmids. The TetRr1.12 plasmids containing the TetRr1.12 cassette in the same orientation as the GFP, pPM3r2.1 (P_{smyc} promoter with 2x $tetO_2$) and pPM4r2.1 (P_{smyc} promoter with 2x $tetO_1$) were linearized and ligated with the HIV-1rt + V3-SV5 epitope tag from plasmid pRC3RT to generate plasmids, pPM3RT and pPM4RT.

4.2.2. INTRODUCTION OF THE HIV-TetRr1.12 PLASMIDS INTO *M. bovis* BCG Δ PANCD

Confirmed HIV-TetRr1.12 plasmids were transformed into BCG Δ panCD. Plasmid DNA (500 ng) was made up to a final volume of 5 μ l in HPLC grade H₂O and electro-transformed into *M. bovis* BCG competent cells as describe in Section 3.2.3.3. The pNM3 plasmid DNA was used as a positive control and 5 μ l HPLC grade H₂O served as a negative control for transformation. In addition to the HIV-TetRr1.12 plasmids, two HIV-TetRr1.7 plasmids, pRC3RT and pRC3Gag provided by Dr Ros Chapman were transformed into BCG Δ panCD. Recombinant mycobacterial colonies were cultured in MB-7H9 media containing appropriate supplements and rBCG Δ panCD[HIV-TetR] (rBCG Δ panCD harbouring the HIV-TetRr1.12 and HIV-TetRr1.7 plasmids) vaccine stocks were made and stored at -80°C as described in Section 3.2.3.4b. The genetic stability of the vaccine stocks was evaluated, by treating the stocks according to the procedure in Section 3.2.3.5 using the restriction enzymes listed in Table 4.1.

Table 4.1: Restriction enzymes used for DNA mapping of the HIV-TetR plasmids

RESTRICTION ENZYMES	RESTRICTED PLASMID	SITE	PRODUCTS (BP)
<i>EcoRV</i>	pPM3Gag	4030, start of the TetRr1.12 gene	4232 and 2451
	pPM4Gag	6431, start of the V3-SV5 epitope tag	4232 and 2452
	pRC3Gag		4232 and 2451
<i>XbaI</i> + <i>HpaI</i>	pPM3RT	4745 (<i>XbaI</i>), start of the <i>P_{smyc}</i>	4856 and 1644
	pPM4RT	promoter	4856 and 1645
	pRC3RT	6389 (<i>HpaI</i>), at the end of the V3-SV5 epitope tag	4856 and 1644

4.2.3. EVALUATION OF THE HIV-TetR PLASMID STABILITY BY DNA SEQUENCING

Plasmid DNA from the rBCG Δ panCD[HIV-TetR] vaccines was isolated as described in section 3.2.3.5, however a large-scale plasmid isolation procedure (Appendix B1.2) was followed. The purified plasmid DNA was then sequenced to confirm genetic integrity. The primer binding sites on the plasmid maps are shown in Figure 4.4. The primer sequences are shown in Table 4.3.

The primers were designed with the aid of a Primer Design software IDT DNA oligoanalyzer and synthesised by Integrated DNA Technologies [Whitehead Scientific]. The sequencing procedure was performed at the University of Stellenbosch [Cape Town] and the sequences viewed with the Chromas v2.3 software package [Technelysium] and edited and aligned against constructed theoretical sequences using DNAMAN bioinformatics software [Lynnon Biosoft, version 4.0.0.1].

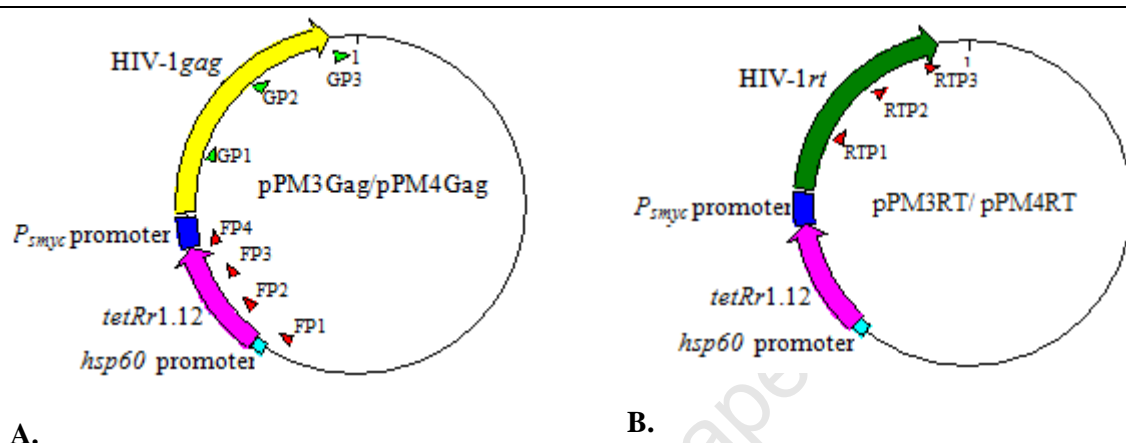


Figure 4.4: An illustration of the primer binding sites on the HIV-TetRr1.12 plasmid maps. A. The GP1, GP2 and GP3 primers were used to sequence the HIV-1 *gag* gene; **B.** The RTP1, RTP2 and RTP3 were used to sequence the HIV-RT gene containing plasmids. The FP1, FP2, FP3 and FP4 primers were used to sequence the *tetRr1.12* gene, the *hsp60* and the *P_{myc}* promoters and the junctions on the HIV-TetRr1.12 plasmids.

4.2.4. ASSESSMENT OF THE REGULATION OF ANTIGEN EXPRESSION IN rBCG Δ PANCD[HIV-TETR]

4.2.4.1. HIV antigen expression in the absence of anhydrotetracycline

rBCG Δ panCD[HIV-TetR] were cultured as described in Section 3.2.3.1, the media contained 10 μ g/ml Kan, 24 μ g/ml vitamin B5 and 25 μ g/ml hygromycin. Once the starter cultures had reached OD₆₀₀ of 0.8 units, they were used to inoculate 100 ml of the same media in 1 L Schott bottles and placed on rollers at 37°C. Cell lysates were extracted from the cultures at 3 different growth phases: early logarithmic (log) (OD = 0.2 - 0.4), mid-log (OD = 0.6) and late log (OD = 0.8) using the SDS boiling method as described in Appendix B5.2. The protein samples were quantified and separated by SDS-PAGE as described in Appendices B6 and B7.2.1, respectively. Laboratory purified proteins, RT and Gag (provided by Dr Nyasha Chin'ombe) were used as positive controls and the rBCG Δ panCD[pCONEPI] cell lysate was used as a negative control.

Proteins were transferred to a PVDF membrane according to Appendix B7.2.2. For detection of the RT protein, the membranes were probed with anti-RT primary antibody (ARP428) [AIDS Reagent Program] at a dilution of 1:5000 and then probed with an anti-goat/sheep monoclonal secondary antibody (A8062) [Sigma], which is linked to the alkaline phosphatase (11697 471 001) [Roche]. For the Gag protein detection, anti-Gag primary antibody (ARP432) [AIDS Reagent Program] at a dilution of 1:5000 was used and probed with a goat anti-rabbit monoclonal secondary antibody (A3687) [Sigma], which is linked to the alkaline phosphatase.

4.2.4.2. Repression and induction of HIV antigen expression in the presence and absence of anhydrotetracycline

This experiment was designed to monitor the differences in HIV-1 antigen expression levels produced by the rBCG $\Delta panCD$ [HIV-TetR] cultured in the presence and absence of ATc. Duplicate starter cultures of rBCG $\Delta panCD$ [HIV-TetR] were prepared as described in Section 3.2.3.1, the media contained appropriate supplements and ATc (2.0 $\mu\text{g/ml}$) was included in order to repress the expression of the HIV-1 antigens. Once the starter cultures had reached OD_{600} of 0.8 units, they were used to inoculate 100 ml of the same media in 1 L Schott bottles. The cultures were grown for a week. To note the repression of HIV-1 antigens, cell lysate was prepared from 25 ml of each culture and analysed on SDS-PAGE and Western Blotting as described in Appendix B7.2.

The remaining cultures were split into two, each centrifuged at 4 000 rpm for 10 min at 4°C and the pellets were resuspended in 100 ml fresh MB-7H9 media and then centrifuged as before. The pellets were resuspended in 100 ml fresh media, one in MB-7H9-Kan containing ATc and the other in MB-7H9-Kan lacking ATc. The cultures were placed back on rollers at 37°C for another week, after which proteins were extracted at the different growth phases as mentioned in Section 4.2.3.1. The protein samples were quantified and analysed by SDS-PAGE and Western Blotting.

4.3. RESULTS

4.3.1. CONSTRUCTION OF THE HIV-T_{ET}Rr1.12 PLASMIDS

The most stable TetRr1.12 plasmids were pPM3r2.1 and pPM4r2.1 for the model antigen GFP (Chapter 3). These plasmids were therefore selected to construct the episomal multicopy HIV-TetRr1.12 systems by removing the *gfp* gene and replacing it with the full length HIV-1 subtype C *rt* and *gag* genes as shown in Section 4.2.1.1 (Figures 4.2 & 4.3).

The HIV-1 *gag* and *rt* gene were derived from those reported by Burgers *et al.*, (2006)²⁷⁴. The HIV-1 genes were linked to the immunodominant V3-SV5 epitope, which was included to compare the cellular immunogenicity of different vaccines in mice in our laboratory (not part of this study). The V3-SV5 epitope consists of the V3-CTL tag that is derived from the HIV-1 subtype B envelope protein, and the paramyxo-virus derived SV5 monoclonal antibody tag. The HIV-1 *rt* gene, *gag* gene and the V3-SV5 epitope tag were codon optimized in our laboratory for efficient expression in mycobacteria (Dr Helen Stutz; Dr William Bourn), synthesized and supplied by GeneArt® (Germany).

Plasmid mapping was performed to screen for positive recombinants as described in section 3.2.2, where small-scale plasmid DNA isolated from *E. coli* clones transformed with the various ligation mixes was subjected to restriction enzyme digests. An *EcoRV* digest was performed to confirm the presence of the *gag* gene, and a double *XbaI* + *HpaI* digest was performed to confirm the presence of the *rt* gene. The mapping results showed that the HIV-1 antigens had been inserted downstream of the *P_{smyc}* promoter generating four new HIV-TetRr1.12 plasmids, which are listed in Table 4.2 (plasmid maps in Appendix E12 - 15).

4.3.2. GENERATION OF RBCG Δ PANCD[HIV-T_{ET}R] EXPRESSING HIV ANTIGENS

The confirmed HIV-TetRr1.12 plasmids, pPM3RT, pPM3Gag, pPM4RT and pPM4Gag and the HIV-TetRr1.7 plasmids, pRC3RT and pRC3Gag were transformed into BCG Δ panCD by electroporation. Plasmid pRC3RT contains the *tetRr1.7* regulator gene orientated in the same direction as the HIV-1 RT gene driven by the full-length *P_{smyc}* promoter with two operator sequences (*tetO₂*) situated on either side of -10. Plasmid pRC3Gag contains the *tetRr1.7* regulator gene orientated in the same direction as the HIV-1 *gag* gene that is driven by the full-

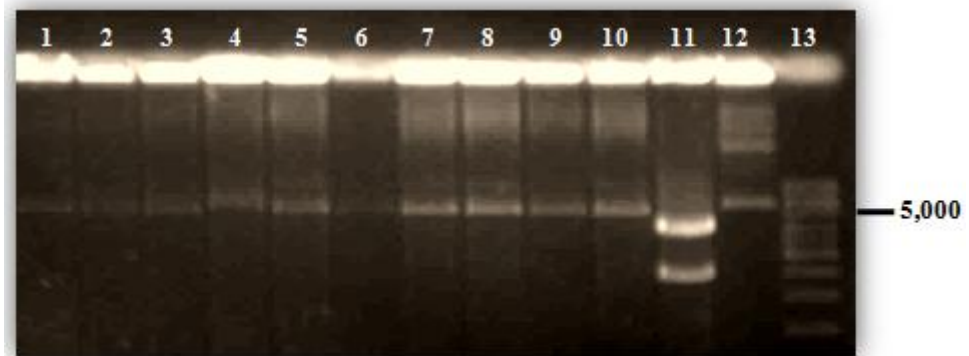
length P_{smyc} promoter with two operator sequences ($tetO_2$) situated on either side of the -10. rBCG $\Delta panCD$ [HIV-TetR] vaccine stocks were prepared in the presence of ATc and plasmid DNA was isolated from the vaccine stocks and evaluated for vector stability by restriction enzyme mapping.

Table 4.2: Names and constitution of the HIV-TetRr1.12 systems

	PLASMID NAME	FEATURES
HIV-TetRr1.12 plasmids	pPM3RT	Plasmid contains the <i>tetRr1.12</i> repressor gene orientated in the same direction as the HIV-1 <i>rt</i> gene that is driven by the full-length P_{smyc} promoter with two operator sequences ($tetO_2$) situated on either side of -10
	pPM3Gag	Plasmid contains the <i>tetRr1.12</i> repressor gene orientated in the same direction as the HIV-1 <i>gag</i> gene that is driven by the full-length P_{smyc} promoter with two operator sequences ($tetO_2$) situated on either side of -10
	pPM4RT	Plasmid contains the <i>tetRr1.12</i> repressor gene orientated in the same direction as the HIV-1 <i>rt</i> gene that is driven by the full-length P_{smyc} promoter with two operator sequences ($tetO_1$) situated on either side of -10
	pPM4Gag	Plasmid contains the <i>tetRr1.12</i> repressor gene orientated in the same direction as the HIV-1 <i>gag</i> gene that is driven by the full-length P_{smyc} promoter with two operator sequences ($tetO_1$) situated on either side of -10

A total of 20 colonies were mapped for each system, but only 10 are shown in the Figures. The plasmids harbouring the *gag* gene were very unstable. As can be seen in Figure 4.5, none of the plasmids produced the expected bands of 4232 bp and 2451 bp after restriction digestion with *EcoRV* as seen in the control lanes. Additionally, no rBCG $\Delta panCD$ [pPM4Gag] transformants could be attained following electroporation, indicating this vector was extremely unstable. Mapping results of the plasmids harbouring the *rt* gene can be seen in Figure 4.6. The genetic integrity plasmids, pPM3RT and pRC3RT was verified since expected bands of 4856 bp and 1644 bp were obtained after restriction digestion with *HpaI* + *XbaI* as seen in the control lanes (panels A and B). However, plasmid pPM4RT was genetically unstable as the expected band patterns were not obtained after the restriction digest with *HpaI* + *XbaI* (panel C).

A.



B.

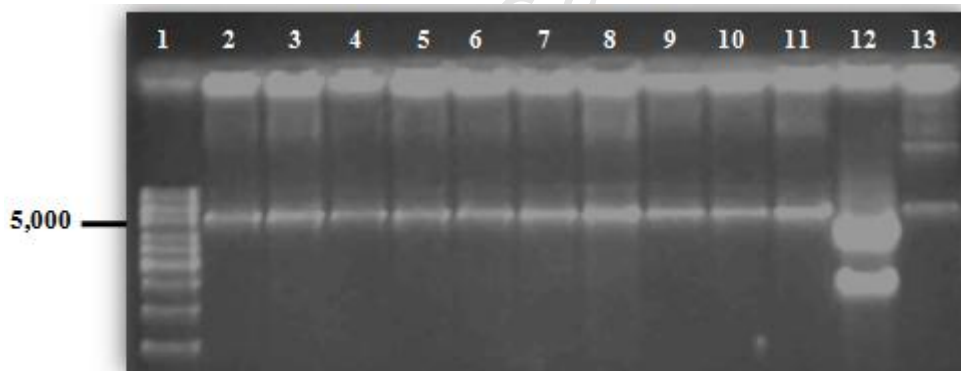
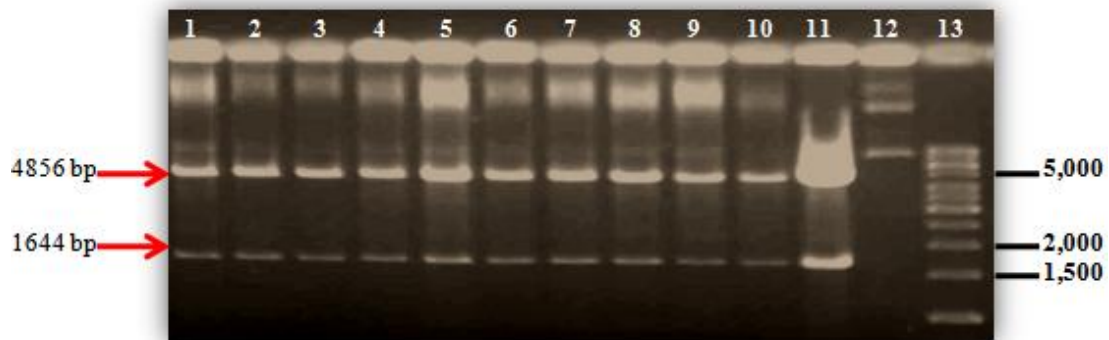
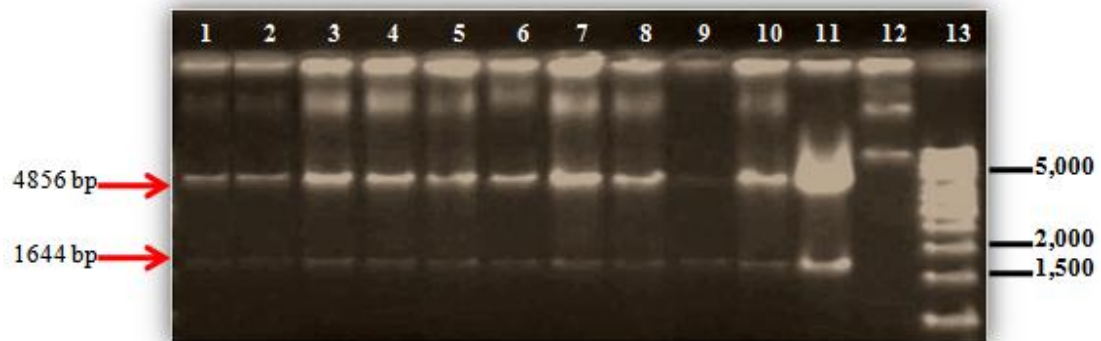


Figure 4.5: Plasmid DNA mapping of the HIV-TetR plasmids harbouring the *gag* gene using *EcoRV*. **A.** pPM3Gag: Lanes: (1 - 10) plasmid DNA isolated from rBCG vaccine stocks; (11) positive control pPM3Gag DNA; (12) uncut pPM3Gag plasmid; (13) 1 kb ladder [promega]. **B.** pRC3Gag: Lanes: (1)1 kb ladder; (2 - 11) plasmid DNA isolated from rBCG vaccine stocks; (12) positive control pRC3Gag DNA; and (13) uncut plasmid. 20 colonies of each system were mapped, but only 10 are shown in the figure.

A.



B.



C.

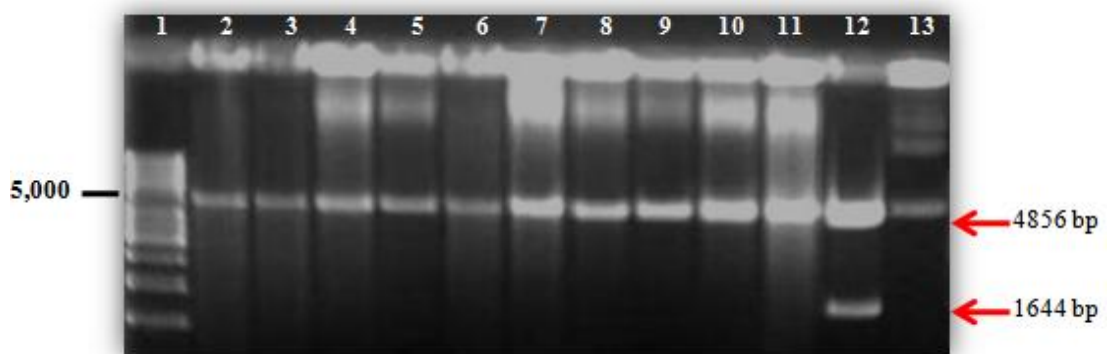


Figure 4.6: Plasmid DNA mapping of the HIV-TetR plasmids harbouring the *rt* gene using *Hpa*I and *Xba*I enzymes. A. pPM3RT: Lanes: (1 - 10) plasmid DNA isolated from rBCG Δ *panCD* vaccine stocks; (11) positive control pPM3RT DNA; (12) uncut pPM3RT plasmid; and (13) 1 kb ladder [promega]. **B.** pRC3RT: Lanes: (1 - 10) plasmid DNA isolated from rBCG vaccine stocks; (11) positive control pRC3RT DNA; (12) uncut pRC3RT plasmid; and (13) 1 kb ladder. **C.** pPM4RT: Lanes (1) 1 kb ladder; (2 - 11) plasmid DNA isolated from rBCG vaccine stocks; (12) positive control pPM4RT DNA; and (13) uncut plasmid DNA. 20 colonies of each system were mapped, but only 10 are shown in the figure.

Sequencing reactions were performed on the plasmid DNA isolated from rBCG Δ *panCD*[HIV-TetR] in order to confirm genetic integrity of the stable plasmids and identify the unstable/deleted regions in the unstable plasmids. The primers used are listed in Table 4.3. Primers RTP1, RTP2 and RTP3 were used to sequence the plasmids harbouring the *rt* gene as these primers span the entire *rt* gene and the restriction sites flanking it. Primers GP1, GP2 and GP3 that span the entire *gag* gene and the restriction sites flanking the gene were used to sequence the plasmids harbouring the *gag* gene. Furthermore a series of four forward primers, FP1, FP2, FP3 and FP4 were used to sequence all HIV-TetR plasmids since these allow for the sequencing of the *tetRr1.12* gene, the *hsp60* and the *P_{smyc}* promoters plus the junctions on the plasmids.

Table 4.3: List of primers used to confirm the integrity of the HIV-TetRr1.12 by DNA sequencing.

TEMPLATE	NAME	POSITION ON THE PLASMID MAP	SEQUENCE
pPM3RT/ pPM4RT	RTP1	5329	5'-CTACTTCTCGGTGCCGC-3'
	RTP2	5769	5'-TCGGCAAGCTGAACTGGA-3'
	RTP3	6186	5'-TCTGGTGGACCGACTACTG-3'
pPM3Gag/ pPM4Gag	GP1	5329	5'-GAAGACCCAGCAGGCCA-3'
	GP2	5929	5'-GAACTGGATGACCGACACC-3'
	GP3	6529	5'-CTTCAAGCAGATCCCGAACC-3'
pPM3Gag/ pPM4Gag	FP1	3821	5'-TGAATATCGTGGAGCTCACC-3'
	FP2	4182	5'-CCTGTACTGGCACGTGAAGA-3'
	FP3	4462	5'-ATCTCGGCCGTGTCGCACTT-3'
	FP4	4729	5'-CTCCTCTGGTTGGTACTCTA-3'

Sequencing confirmed the genetic integrity of the plasmids, pPM3RT and pRC3RT as output showed complete sequence identity to the sequences designed by the manufacturer (GeneArt®). Deletions on the unstable plasmids were identified. A 1170 bp fragment containing p24, p6 and the V3-SV5 epitope tag (Appendix D4&5) was deleted from the plasmids, pPM3Gag and pRC3Gag, and a 1452 bp fragment containing the *rt* gene and the V3-SV5 epitope tag (Appendix D6) was deleted from the pPM4RT plasmid. The restriction sites *EcoRV* (6431) on the plasmids harbouring *gag* and *HpaI* (6572) on plasmids harbouring *rt*, lie within the deleted regions, and thus were lost. This data explains the unexpected band patterns produced following restriction digests of the plasmids (Figures 4.5 and Figures 4.6 C).

4.3.3. THE EXPRESSION OF THE HIV-1 ANTIGENS IN rBCG Δ panCD[HIV-TetR] IN THE PRESENCE AND ABSENCE OF ANHYDROTETRACYCLINE

HIV-1 antigen expression levels were investigated by Western blot analysis. Initially antigen expression was evaluated in the absence of ATc. rBCG Δ panCD were cultured without ATc and the cell lysates were extracted from the cultures at early log growth phase (OD = 0.2 - 0.4), mid-log growth phase (OD = 0.6) and late log growth phase (OD = 0.8). Protein expression levels were analysed from the extracted lysates. Figures 4.7 and 4.8, respectively depict Western blot results from the rBCG Δ panCD[HIV-TetR] systems displaying the expressed Gag and RT proteins. Without ATc a small quantity of Gag protein was produced by rBCG Δ panCD[pPM3Gag] and rBCG Δ panCD[pRC3Gag] during early log and mid-log growth phases, however there was no visible Gag protein detected during late log growth phase (Figure 4.7). As expected the negative control, rBCG Δ panCD [pCONEPI] did not express any HIV protein and the positive control, purified HIV-1 Gag produced a 56 kD protein.

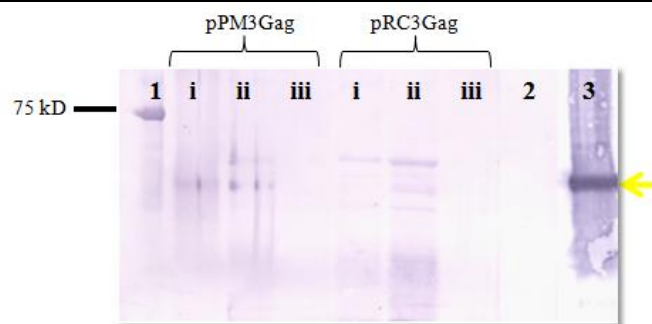


Figure 4.7: Detection of the Gag protein from rBCG Δ panCD[HIV-TetR] cell lysate extracted during different growth phases in the absence of ATc. Roman letters represent the growth phases, i. Early log; ii. Mid-log; iii. Late log. Lanes 1. Dual color standard [Biorad]; 2. Negative control rBCG Δ panCD[pCONEPI] cell lysate; 3. Positive control, purified HIV-1 Gag (60 ng/ml). The ARP 432 anti-Gag antibody [Sigma] was used to detect the Gag proteins. The yellow arrow represents the HIV-1 Gag protein.

Similarly the recombinants harbouring *rt*, rBCG Δ panCD[pPM3RT] and rBCG Δ panCD[pRC3RT] showed detectable RT protein levels during the early growth phases, which was lost during culturing as no antigen was detected during the late-log phase (Figure 4.8). rBCG Δ panCD[pPM4RT] on the other hand displayed very high instability as no detectable levels of RT were seen. The positive and negative controls gave expected results, rBCG Δ panCD [pCONEPI] negative did not produce any detectable HIV proteins and the purified HIV-1 RT that served as a positive showed a very clear band of expected size (49 kD).

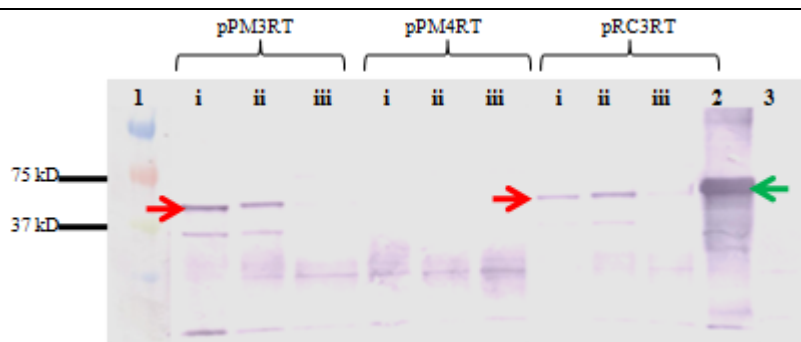
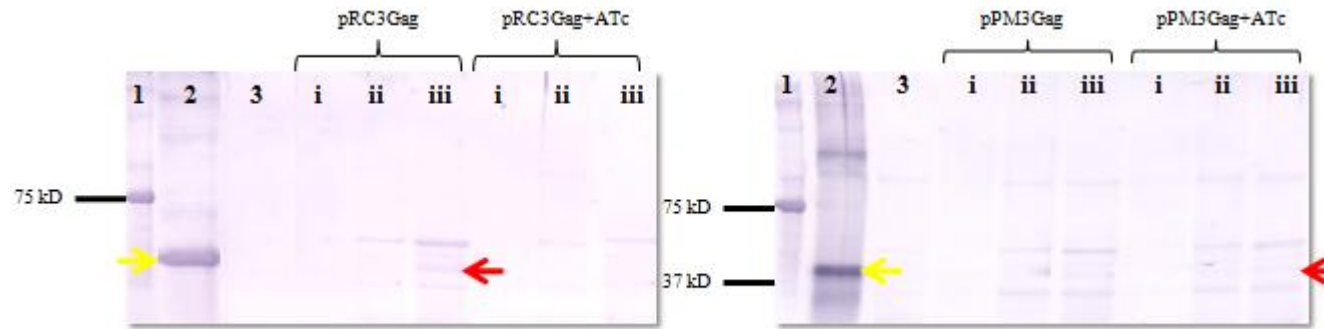


Figure 4.8: Detection of the RT protein from rBCG $\Delta panCD$ [HIV-TetR] cell lysate extracted during different growth phases in the absence of ATc. Roman letters represent the growth phases, i. Early-log; ii. Mid-log; iii. Late-log. Lanes 1. Kaleidoscope [Biorad]; 2. positive control, purified HIV-1RT (60 ng/ml) shown by the green arrow; 3. Negative control rBCG $\Delta panCD$ [pCONEPI] cell lysate. ARP 428 anti-RT antibody [Sigma] was used for detection. The red arrows represent the HIV-1 RT proteins and the green arrow indicates the purified HIV-1 RT protein, used as a positive control.

Expression of the HIV-1 antigens was then monitored in the presence of ATc in order to assess if regulating the expression improved the retention of the HIV-1 genes. The rBCG were grown with ATc and the HIV-1 antigen expression was noted. Subsequently the cultures were divided into two. One culture was washed to remove ATc and the induction rate was noted to determine the time point at which the highest levels HIV-1 antigens were produced. The other culture was not washed but was also monitored from the early to the late log growth phase.

Repression of the HIV-1 antigen expression was observed in the presence of ATc as shown by the faint bands detected at the early log growth phase (Figure 4.9). However, the systems were not completely repressed, which is similar to the results obtained in Chapter 3, where the rBCG $\Delta panCD$ protein expression was kept at low levels in the presence of ATc. A gradual induction was observed after the removal of the repressor, from the washed cultures, from mid-log to late-log growth phase. The highest levels of protein production were observed at the late-log growth phase. Additionally, the cultures that were not washed (ATc⁺), displayed low levels of protein expression during early log phase growth, which increased slightly with time, this could have resulted from the depletion of ATc molecules as the culture density was increasing or ATc may have reached its half-life. Some protein bands larger than the RT protein (Figure 4.9 B) were seen. These bands were probably due to non-specific binding of the primary antibody to mycobacterial proteins in the BCG cell lysate. This could be due to the use of Freund's complete adjuvant in the generation of the anti-RT antibody. Freund's complete adjuvant contains mycobacterial proteins

A.



B.

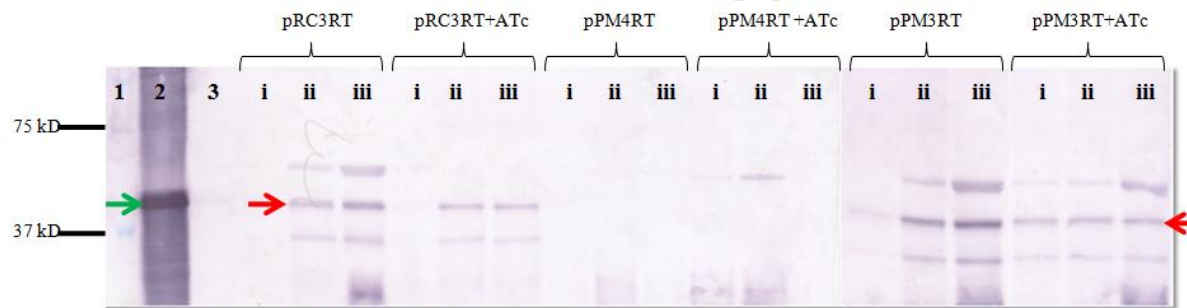


Figure 4.9: Detection of HIV-1 antigens from rBCG $\Delta panCD$ [HIV-TetR] cell lysate extracted during different growth phases. Roman letters represent the growth phases, i. Early-log; ii. Mid-log; iii. Late-log. A. Detection of the Gag protein using the ARP 432 anti-Gag antibody [AIDS Reagent program]: Lanes 1. Dual color standard [Biorad]; 2. Positive control, purified HIV-1 Gag (60 ng/ml) shown by the yellow arrows; 3. Negative control, rBCG $\Delta panCD$ [pCONEPI] cell lysate. B. Detection of the RT protein using the ARP 428 anti-RT antibody [AIDS Reagent program]: Lanes 1. Kaleidoscope [Biorad]; 2. Positive control, purified HIV-1 RT (60 ng/ml) shown by the green arrow; 3. Negative control, rBCG $\Delta panCD$ [pCONEPI] cell lysate. The red arrows represent the HIV-1 RT and Gag proteins.

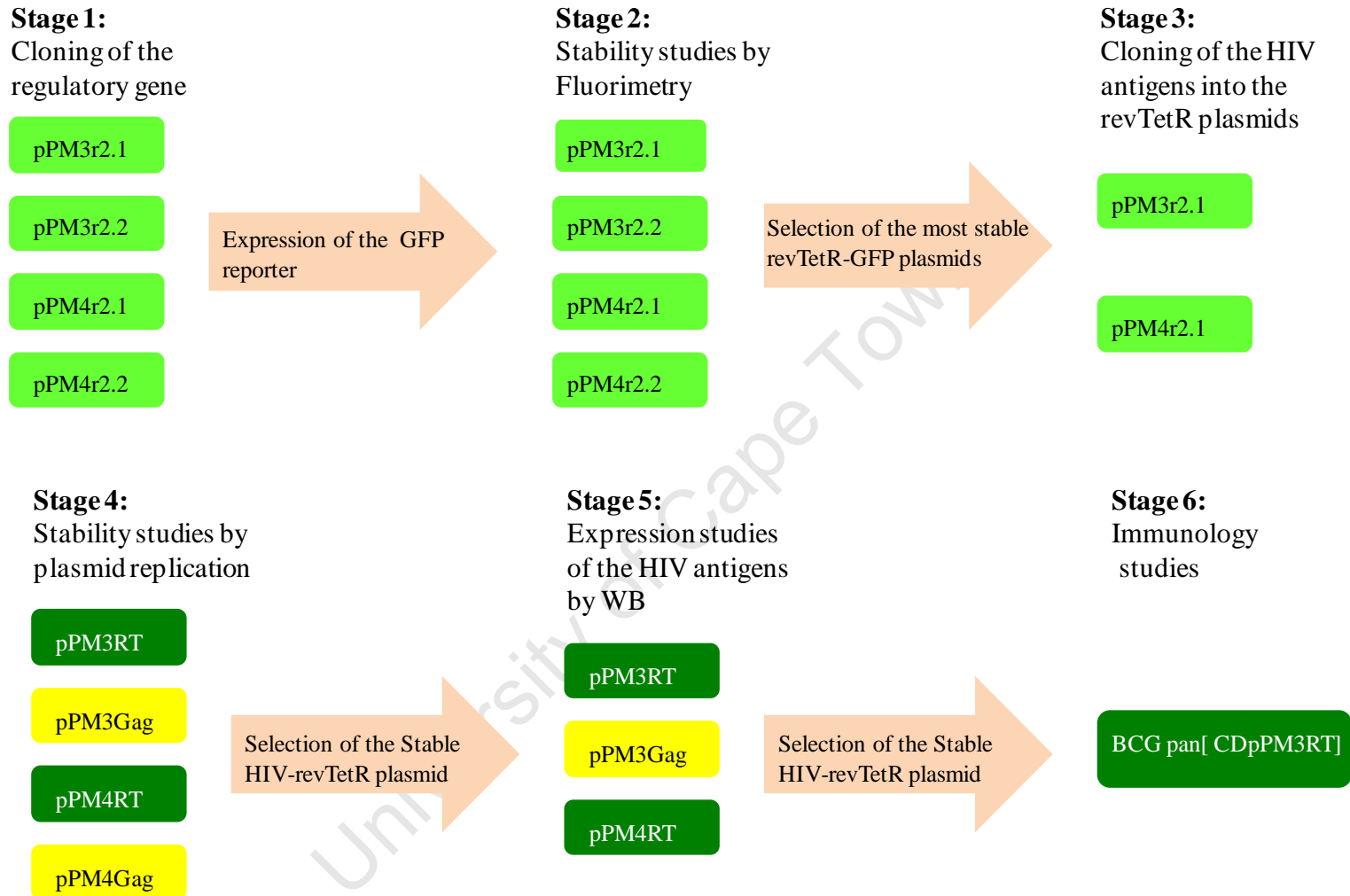


Figure 4.10: Overall summary of the construction and evaluation of the expression systems, from the episomal TetRr1.12 regulatory systems to the multicopy HIV-TetRr1.12 systems. The plasmids named with pPM3 prefix contain the full-length P_{smyc} promoter with two $tetO_2$ sequences situated on either side of -10 and those named with pPM4 prefix contain the full-length P_{smyc} promoter with two $tetO_1$ sequences situated on either side of -1.

4.4. DISCUSSION

We have successfully constructed the episomal multicopy HIV-TetRr1.12 expression systems by cloning the HIV-1 subtype C, *rt* and *gag* genes into the TetRr1.12 regulatory systems. The HIV-TetRr1.7 constructs, pRC3RT and pRC3Gag were also included in the study. In all the HIV-TetR systems the HIV-1 antigens were expressed from the inducible P_{smyc} promoter containing the *tetO* operator sequences, which allowed for the control of expression *in vitro*. All HIV-TetR plasmids maintained genetic stability in *E. coli*, however in some of the vectors structural instability occurred after introduction into BCG $\Delta panCD$, even in the presence of the ATc. The unstable plasmids were pPM3Gag, pRC3Gag and pPM4RT.

DNA Sequencing of the unstable plasmids revealed that the HIV-1 antigens were truncated and the V3-SV5 epitope was completely removed from the plasmids, thus these systems were not used for immunogenicity assays. A similar incidence of instability was reported by Joseph *et al.*, (2010)¹²⁵ when they generated an rBCG expressing HIV-1 *gp120* gene from a strong *hsp60* promoter. They observed deletions in the HIV-1 *gp120* gene which resulted in loss of an immunodominant CTL epitope. The stability of the rBCG vaccine was improved by using a weak promoter. They hypothesized that the loss of the immunodominant epitopes represent part of an adaptation process of the mycobacteria to survive in the hosts as this could assist it to escape the host's immune response.

Evaluation of the antigen expression showed that Gag was produced at lower levels compared to RT, which was not surprising given the instability of the plasmids expressing Gag. Low production levels of Gag have been reported in other studies^{107, 182}. The instability of the Gag antigen could be attributed to the complex nature of the Gag protein and the requirement for post-translational processing, which would not occur in rBCG $\Delta panCD$. The bacteria might not have the necessary machinery to fold the Gag protein correctly, which could result in the build-up of misfolded proteins within the cells that might be detrimental.

The presence of ATc improved the stability of rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT], which in turn improved the expression of RT (Figure 4.8). The highest RT levels were obtained during the late-log growth phase, a phase at which no HIV antigens were detected in the absence of ATc (due to instability).

As mentioned in Chapter 1, generally instability of the vaccine vectors occurs in episomal vectors expressing viral antigens from strong promoters. However instability was not expected in our systems in the presence of ATc since the expression of HIV antigens was down-regulated and the data obtained in Chapter 3, indicated that although low levels of GFP expression were observed in the presence of 2.0 µg/ml ATc, the recombinants maintained genetic stability. The basal expression of the HIV antigens caused by incomplete switch-off of the P_{smyc} promoter by 2.0 µg/ml ATc (Figure 4.9) could account for instability of the vectors in this case.

In the induction kinetics study we observed that the levels of the HIV-1 antigens produced by the stable recombinants after ATc was removed were similar to those produced by the stable recombinants that were not induced (Figure 4.9). This was unexpected as we expected the rBCG $\Delta panCD$ containing ATc to remain repressed. This may have been caused by insufficient ATc in the cultures, which may have resulted from the dilution of ATc molecules as the culture density was increasing or ATc may have reached its half-life.

Overall, the presence of ATc improved the stability of the rBCG $\Delta panCD$ expressing the HIV-1 RT antigen. In addition, there was no significant difference in antigen expression levels between the rBCG $\Delta panCD$ harbouring the HIV-TetRr1.12 systems and those harbouring the HIV-TetRr1.7 systems.

CHAPTER 5: IMMUNOGENICITY STUDIES OF *r*BCG HIV-1 SUBTYPE C VACCINE IN BALB/*c* MICE

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5.1. INTRODUCTION

The current study explored the use of a controllable rBCG expressing the HIV-1 RT antigen as an effective vaccine to induce a protective immune response. In the previous chapter we generated various designs of an episomal multicopy HIV-1-TetR system that harbour the HIV-1 RT antigen under the control of an inducible P_{smyc} promoter. The HIV-1-TetR systems contain the components of the TetR system to stringently regulate expression of the HIV antigens. This regulation mechanism is ATc-dependent, such that if ATc is added to the culture, it binds to the TetR repressor and forms the ATc-TetR complex. The formation of the complex changes the structural conformation of the TetR repressor, which enables it to bind to the P_{smyc} promoter on the *tetO* operator sequences. The occupation of the *tetO* operators shuts down the P_{smyc} promoter activity and thus the expression of the antigen is suppressed. This repression of the HIV-1 antigen expression prevents the occurrence of metabolic burden during *in vitro* preparation of the vaccine stocks. The repression is then removed by washing ATc from the cultures to detach it from the TetR repressor. These results in the dissociation of the ATc-TetR complex from the *tetO* operators and subsequently the P_{smyc} promoter is activated which results in HIV-1 antigen expression.

Stability analysis of the HIV-1-TetR systems indicated that the rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] were the most stable systems. Consequently these were selected to generate rBCG HIV-1 subtype C vaccines for immunogenicity studies. The repression of the HIV-1 RT antigen was mediated by the TetRr1.12 repressor in rBCG $\Delta panCD$ [pPM3RT] and by the TetRr1.7 repressor in rBCG $\Delta panCD$ [pRC3RT].

Hypothetically this rBCG vaccine invention will enable the vaccines to remain stable *in vitro* and *in vivo*, and provide the host with an adequate HIV antigen load after vaccination to elicit an effective immune response. The implementation of the TetR system concept in rBCG HIV vaccine development is novel and intended to contribute effectively towards an rBCG HIV-1 subtype C vaccine development.

5.1.1. STUDY OBJECTIVES

Two sets of rBCG $\Delta panCD[pPM3RT]$ and rBCG $\Delta panCD[pRC3RT]$ vaccine stocks were prepared, the first was the induced batch, whereby ATc was removed to up-regulate the expression prior to vaccination. The second batch was the uninduced, whereby ATc was not removed. The objective was to investigate and compare the immune response of BALB/c mice to these vaccines for providing usefulness of the concept of the HIV-1-TetR system-based strategy. The mice were primed with the rBCG vaccines and boosted with a recombinant pox virus vaccine expressing a matching antigen. The experimental plan is depicted graphically in Figure 5.1.

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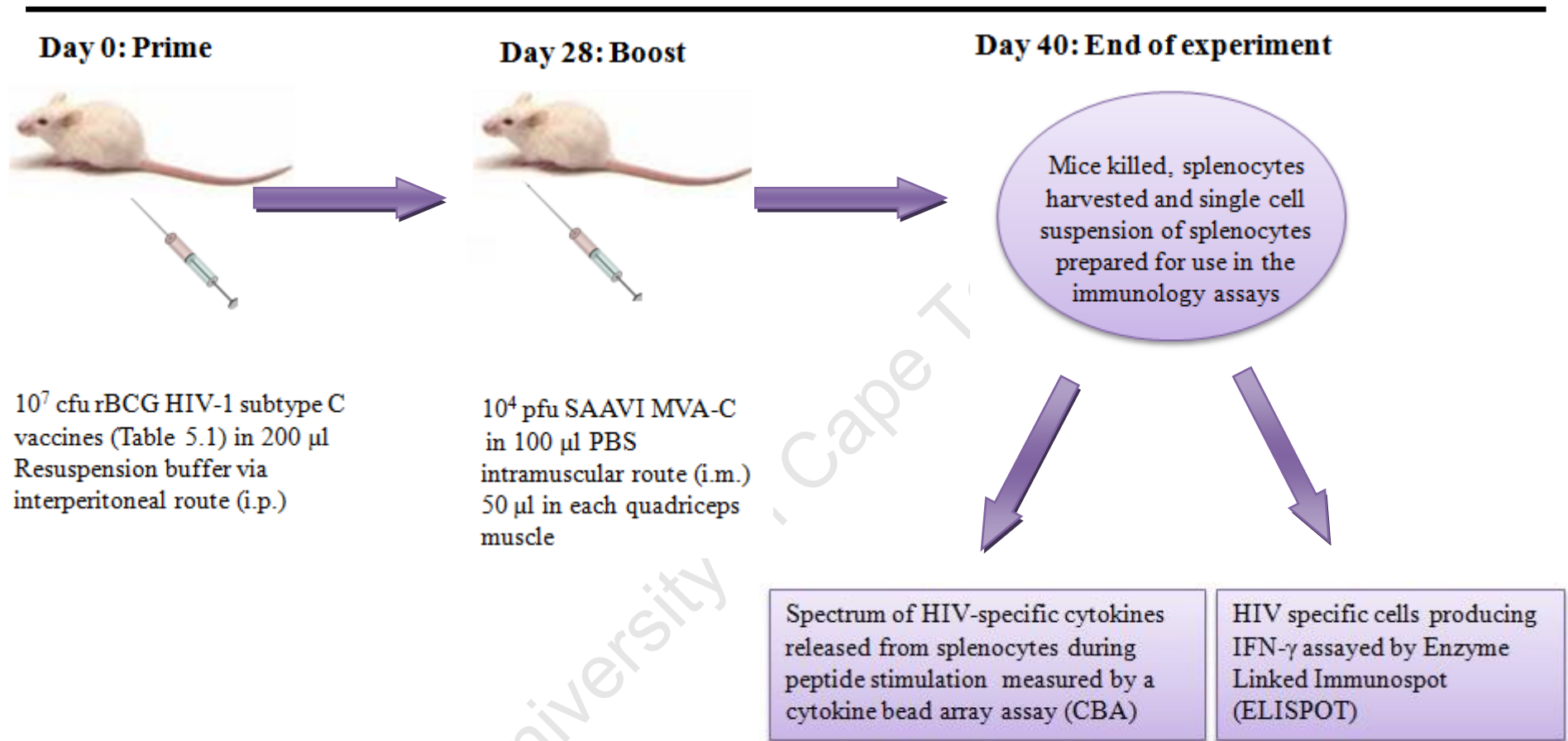


Figure 5.1: A schematic illustration of the vaccination regimen of BALB/c mice: rBCG HIV-1 subtype C vaccine prime, SAAVI MVA-C boost. The vaccines are listed in Table 5.1.

5.2. MATERIALS AND METHODS

5.2.1. PREPARATION OF rBCG HIV-1 SUBTYPE C VACCINE STOCKS

The stable rBCG $\Delta panCD$ [HIV-TetR] systems harbouring pPM3RT and pRC3RT plasmids (Appendix E14 & 15) generated in chapter 4 were used to prepare vaccine stocks. Cultures of rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] were prepared in selective MB-7H9 media containing 10 $\mu\text{g/ml}$ Kan, 2 $\mu\text{g/ml}$ ATc, 24 $\mu\text{g/ml}$ vitamin B5 and 25 $\mu\text{g/ml}$ hygromycin. A week later the cultures (OD_{600} of 0.8) were centrifuged at 4 000 rpm for 10 min at 4°C and the pellets were resuspended in fresh MB-7H9 media and centrifuged as before, this step was performed to wash off ATc. The pellets were then resuspended in 100 ml fresh media either with or without ATc. The cultures were placed back on rollers at 37°C, grown to an OD_{600} of approximately 0.6 (mid to late - log growth phase). The rBCG HIV-1 subtype C vaccine stocks were then prepared by centrifuging the cultures at 4 000 rpm for 10 min at 4°C and resuspending the pellets in 5 ml resuspension buffer. The absorbance adjusted to a final OD_{600} of 10 using resuspension buffer. Aliquots of the vaccine stocks were frozen at -80°C. Vaccine stocks of rBCG $\Delta panCD$ [pCONEPI] were prepared as a negative control. In addition, a multi-gene HIV-1 subtype C recombinant MVA vaccine, SAAVI MVA-C was used as a vaccine boost^{289, 290}. The vaccines are listed in Table 5.1.

Prior to vaccination, aliquots of the vaccine stocks were defrosted on ice, and diluted to the required dose dilutions with resuspension buffer. Vaccine dose was based on the absorbance readings. One absorbance unit was taken to be equivalent to 1×10^7 cfu/100 μl bacteria⁹⁹.

5.2.2. VACCINATION REGIME FOR BALB/C MICE: rBCG $\Delta PANCD$ PRIME - SAAVI MVA-C BOOST

The female BALB/c mice (4 - 6 weeks of age) were purchased from the South Africa Vaccine Producers Pty Ltd (Johannesburg, South Africa). They were housed in the University of Cape Town Animal Unit (University of Cape Town, South Africa) for 10 days to acclimatise before vaccination. Vaccination and further handling were performed by Mr R. Lucas. Ethics approval for the experiments was obtained from the University of Cape Town Research Ethics Committee (Approval number: ref 007/017A).

Table 5.1: Names and features of the vaccine vectors used to vaccinate BALB/c mice

VACCINE VECTORS	FEATURES
rBCG ΔpanCD[pPM3RT]	Plasmid contains the <i>tetRr1.12</i> regulator gene orientated in the same direction as the HIV-1 RT gene that is driven by the full-length P_{smyc} promoter with two operators (<i>tetO₂</i>) sequences situated on either side of -35
rBCG ΔpanCD[pRC3RT]	Plasmid contains the <i>tetRr1.7</i> regulator gene orientated in the same direction as the HIV-1 RT gene that is driven by the full-length P_{smyc} promoter with two operators (<i>tetO₂</i>) sequences situated on either side of -35
rBCG ΔpanCD[pPM3RT]+	rBCG Δ panCD[pPM3RT] cultured with ATc
rBCG ΔpanCD[pRC3RT]+	rBCG Δ panCD[pRC3RT] cultured with ATc
rBCG ΔpanCD[pCONEPI]	Empty vector control, expressing no HIV antigen
SAAVI MVA-C	A double recombinant, produced by inserting <i>Grtn</i> (HIV-1 subtype C genes, <i>gag</i> , <i>reverse transcriptase</i> , <i>tat</i> , and <i>nef</i>) and <i>gp150</i> (a truncated HIV-1 subtype C <i>env</i>) into two different sites in MVA. The Env protein has a C-terminus BALB/c mouse CD8 epitope from the HIV-1 subtype B envelope protein (V3-CTL) with amino acid sequence which corresponds to the amino acid sequence of the NIH CD8 tag on the rBCG vaccines expressing the RT antigen ²⁷⁴

For the experiments, mice were divided into 6 groups with 5 mice per group, and 5 groups were each given a rBCG Δ panCD vaccine or control vaccine (Table 5.1) at a dose of 1×10^7 cfu in 200 μ l of resuspension buffer via intraperitoneal injection on Day 0. The mice were boosted 28 days later with 10^4 plaque forming units (pfu) SAAVI MVA-C in 100 μ l PBS [Gibco], with 50 μ l injected into each quadriceps muscle. The sixth group of the mice was vaccinated with SAAVI MVA-C only at a dose of 10^4 pfu in 100 μ l PBS, with 50 μ l injected into each quadriceps muscle on day 28. The experiment was ended on day 40. All mouse groups were killed by cervical dislocation without anaesthesia, the spleens were harvested and five spleens from the same group were pooled for isolation of a single cell suspension of splenocytes.

5.2.3. IMMUNOGENICITY ASSAYS

5.2.3.1. Preparation of mouse splenocytes

The following procedure was performed to generate a single cell suspension of splenocytes from the spleens under sterile conditions. The spleens (1 spleen/10 ml RPMI [Gibco]) were poured into a cell strainer (70 μ m) placed in a petri dish and mashed with a 2 ml syringe rubber plunger [Sigma]. The cell suspension was transferred to a 50 ml centrifuge tube [Falcon]. The petri dish was washed with 10 ml RPMI to collect residual cells, then added to the cell suspension. The cell suspension was made up to 50 ml with RPMI and centrifuged at 1 500 rpm for 5 min to pellet the cells. The supernatant was discarded and the cell pellet was resuspended in 50 ml of RPMI. The cells were then washed twice with 50 ml of RPMI (before centrifuging the cells at the last wash, fibrin clots were removed using a pasture pipette to ensure that any extracellular matter was eliminated). The supernatant was discarded and the pellet resuspended in a final volume of 50 ml RPMI. The red blood cells (RBC) were lysed by addition of 1 ml of the RBC lysis buffer (Appendix C3) to the 1 ml of splenocyte suspension. The suspension was mixed gently, centrifuged as before, and then the supernatant was removed and the cell pellet was resuspended in 1 ml R10 medium (Appendix C1).

Cell counts were performed and viability determined as follows: a 1:10 dilution of the suspension was made in Trypan Blue (T8154) [Sigma] and Turks (Appendix C2), and counted in a Neubauer counting chamber [Superior, Germany]. The cell concentration in the suspension was calculated and adjusted to the appropriate cell concentration required for Enzyme Linked Immunospot (ELISPOT) assay and Fluorescence-activated cell sorting (FACS) by adjusting the volume.

5.2.3.2. Detection of IFN- γ secreting HIV specific splenocytes using ELISPOT assay

An IFN- γ ELISPOT assay was used to determine the frequency of HIV-specific cells producing IFN- γ in the splenocyte population in response to stimulation with the MHC class II-restricted CD4 peptide (RT-CD4), the H-2Kd-restricted RT peptide (RT-CD8) and V3-CTL CD8 (from HIV-1 subtype B) peptides (Table 5.2).

5.2.3.3. Coating ELISPOT plates with the capture antibody

The IFN- γ assay was carried out using the BD Biosciences kit according to the manufacturer's manual. The 96 well plates [BD Biosciences, USA] were pre-wetted with 35% Ethanol (15 μ l/well) for 60 seconds and then washed three times with 200 μ l of PBS. The plates were coated with capture antibody (anti-IFN- γ at 5 μ g/ml in PBS), 100 μ l of the diluted antibody was added per well. The plates were then sealed and incubated overnight at 4°C. The coating antibody was discarded and the wells were washed once with 200 μ l/well blocking solution (Appendix C2). Blocking solution (200 μ l/well) was added and the plates incubated for 2 hrs at room temperature.

Table 5.2: Stimulants and peptides used in the ELISPOT assay (all peptides were > 95% pure from Bachem)

STIMULANT	CONCENTRATION/ REACTION	DESCRIPTION	PEPTIDE SEQUENCE
R10 medium only	N/A	No antigen (background response)	N/A
Irrelevant peptide	2 μ g/ml	Negative peptide control	-H-TXSTVASSL-OH-
V3-CTL CD8 peptide	2 μ g/ml		-RGPGRAFVTI-
RT-CD8 peptide	2 μ g/ml		-VYYDPSKDLIA-
RT-CD4 peptide	2 μ g/ml		-PKVKQWPLTEVKIKAL TAI-
BCG Pasteur lysate	5 μ g protein/ml	Response to vector	N/A
ConA^a	1 μ g/ml	Non-specific polyclonal stimulus positive control	N/A

^a **ConA:** Concavalin A [Sigma, MO, USA]

BCG Pasteur lysate: [Appendix B5.1]

N/A: Not applicable

5.2.3.4. Plating of the splenocytes and peptides

After blocking for 2 hr, the blocking solution was discarded and the wells were plated in triplicate with splenocytes (100 μ l/well of the cell suspension, that is, 0.5×10^6 cells/well). The peptides and other stimulants listed in Table 5.2 were added (100 μ l/well) to triplicate wells. A

lysate of BCG was used to determine responses to the vector. Concavalin A (ConA) was used as a positive control and the irrelevant peptide was used as a negative control.

The plates were then covered with the lids and aluminium foil and incubated at 37°C with 5% CO₂. After 24 hr incubation, the IFN- γ ELISPOT plates were processed to detect IFN- γ forming units. The well contents were discarded and wells were washed twice with 200 μ l/well of deionized H₂O (wells were allowed to soak for 3 - 5 minutes at each wash step). Wells were washed three times with wash buffer I (Appendix C3) (the washes were programmed on the ELx50 Auto Strip Plate Washer [Analytic and Diagnostic Products, SA]). Excess wash buffer from the wells was removed by blotting the plate on a piece of paper towel.

5.2.3.5. Plate development

To detect spots, the detection antibody, biotinylated anti-IFN- γ was diluted in dilution buffer (Appendix C3) to 2 μ g/ml and added to the plates at 100 μ l/well. The plates were sealed and incubated at room temperature for 2 hrs. The detection antibody solution was then discarded and the wells were washed three times with Wash buffer I. Avidin-horseradish peroxidase (Avidin-HRP) [BioCom Biotechnology, SA] was diluted 1:100 in dilution buffer and 100 μ l/ well was added to the plates. The plates were incubated for 1 hr at room temperature. The avidin-HRP solution was discarded and the plates were washed three times with Wash buffer I followed by another three washes with Wash buffer II (Appendix C3). The Nova Red Substrate solution (Appendix C2) was prepared and added to the plates, 100 μ l/well. The spots were allowed to develop for 5 - 10 min in the dark. The development of the spots was stopped by washing the cells with cold running tap water (5 - 6 washes). The plates were air-dried at room temperature overnight and stored in the dark until analysis.

The plates were scanned using the ELISPOT CTL Analyzer [Series 3B) and the number of spots enumerated using computerized Immunospot Image Analyzer [Cellular Technology Ltd] with Immunospot Version 3.2 software. For each reaction, the mean number of spots from triplicate wells and standard deviation (SD) of this mean were calculated and adjusted to spot forming units (sfu)/10⁶ splenocytes. The mean number of spots and its SD in the absence of peptide or in the presence of an irrelevant peptide for each group was used as a background response for the group and were subtracted from the response to the HIV-1 peptides. Responses of the splenocytes to the HIV-1 peptides in each group were considered positive if \geq 1.5 fold the

individual peptide response for a prime with the control BCG $\Delta panCD$ and SAAVI MVA-C boost.

5.2.4. QUANTIFICATION OF CYTOKINES SECRETED BY SPLENOCYTES USING CYTOKINE BEAD ARRAY (CBA) ASSAY

The CBA employs a number of inert particles with different fluorescent intensities that are coated with specific anti-cytokine antibodies and can be used simultaneously to detect multiple cytokines in a single specimen. The splenocytes (1.5×10^6 cells/299 μ l/well) were cultured with the HIV-1 peptides (Table 5.2) for 48 hrs and the cytokine content of the extracellular supernatant was determined using a Mouse Inflammation Cytokine Bead Array (CBA) kit [BD Pharmingen] according to manufacturer's manual that allowed for detection of the amount of IFN- γ , TNF- α , IL-6, IL-10, MCP-1, and IL-12p70 released during culture of splenocytes with the RT-CD8 peptide, the RT-CD4 peptide or the V3-CTL CD8 peptide.

A six bead population with distinct fluorescence intensities have been coated with capture antibodies specific for the proteins listed above and mixed together to form the CBA, which is resolved in the FL3 channel of the BD FACSCaliburTM flow cytometer. Cytokine standards from 0 - 5000 pg cytokine/ml that were provided in the kit were prepared for each cytokine by serial dilutions. Capture beads (50 μ l/sample) were added to the appropriate test or standard tubes. Test sample or standard, 50 μ l, was then added to the capture beads, followed by the addition of 50 μ l of the Mouse Inflammation PE detection reagent. The assay tubes were incubated for 2 hrs at room temperature in the dark. A volume of 1 ml of Wash buffer I (Appendix C3) was added to each assay tube and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the bead pellets suspended in 300 μ l of the wash buffer I. The samples were vortexed and analyzed on the BD FACSCalibur[®] [BD BioSciences, USA] with the BD CBA software. The standard curves for the cytokines were then generated. The results were calculated as pg cytokine per 10^6 splenocytes.

Background cytokine responses in the absence of peptide or in response to the irrelevant peptide were not more than 5 pg per 10^6 splenocytes. Cytokine values obtained with the individual peptide stimuli for the prime-boost regimen were considered positive if ≥ 1.5 -fold above the response for a prime with the control BCG $\Delta panCD$ and SAAVI MVA-C boost.

5.2.5.DETERMINATION OF LYMPHOCYTE PHENOTYPES IN THE SPLENOCYTE POPULATION POST VACCINATION USING FLOW CYTOMETRY

T and B cell proportions in the splenocyte population were determined using specific fluorescent antibodies that bind to receptors on B- and T-cells. The detection was done using flow cytometry. Cell surface receptors were stained with a fluorescent antibody mix listed in Table 5.3. A suspension of 1×10^6 splenocytes were added into a 50 ml skirted tube [Falcon], 25 μ l blocking solution was added to each tube and mixed. The tubes were then incubated on ice for 20 min. The cells were washed with 1 ml FACS buffer (Appendix C3) and centrifuged at 1500 rpm for 5 min. The supernatants were discarded and the cells were resuspended in the residual FACS buffer (small amount of FACS buffer left in the tubes after centrifugation).

A volume of 50 μ l of the fluorescent antibody mix was added into each tube, mixed and incubated on ice in the dark for 30 min. The cells were then washed twice in 2 ml of the FACS buffer and resuspended in the residual volume of the FACS buffer. FACS buffer (400 μ l) was added to each tube and then the cells were acquired using a BD FACSCalibur® [BD BioSciences, USA]. The data was analysed using DB Cell-quest Pro software.

Table 5.3: Fluorescing Antibodies used to detect cell surface receptors.

ANTIBODIES	DILUTION IN FACS BUFFER
CD3-APC	1:50
CD8-PerCP	1:50
CD4-FITC	1:400
CD19-PE	1:50

These antibodies were previously titrated and confirmed to stain optimally at the following dilutions.

5.2.6. RECOVERY OF rBCG Δ PANCD FROM MOUSE SPLEENS AND DETECTION OF THE PLASMID INSERT

Following plating for the ELISPOT assay, rBCG present in the remaining splenocyte suspensions was grown and assessed for the presence of the plasmid used to transform the bacteria. For this the splenocytes from Section 5.2.3 were centrifuged at 1 200 rpm for 10 min and diluted 1:1 and 1:2 in resuspension buffer. These samples were then plated on MB-7H10 agar (containing 24 μ g/ml vitamin B5, 25 μ g/ml hygromycin and 10 μ g/ml Kan. The plates were incubated at 37°C for 3.5 - 4 weeks.

From the resulting rBCG Δ panCD colonies, 5 colonies were picked from each vaccination group. The rBCG Δ panCD colonies were mechanically lysed to release plasmid DNA using the Fastprep procedure. Approximately 100 μ l of Silica/Zirconia beads (0.1 mm) [BioSpec] were added to the bottom of the 2 ml screw top eppendorf tubes [BioSpec]. A single colony of rBCG Δ panCD resuspended in 100 μ l of HPLC H₂O was added to the beads and mixed. The tubes were placed in a Fastprep machine [Southern Cross Biotechnology] and spun at speed 6 for 40 sec (the step was repeated twice). The contents were then centrifuged at 14 000 rpm for 5 min and the supernatant, containing plasmid DNA was transferred into a new 1.5 ml eppendorf tube [Merck].

Plasmid DNA was electroporated into *E. coli* as described in Appendix A5. Four colonies from each transformations were picked off the plates and cultured as described in Section 3.2.2.1. Thus, 20 plasmid DNA samples were generated from each splenocyte suspension (vaccine group). The small-scale plasmid DNA was isolated from the cultures according to Appendix B1.1 and restriction enzyme mapping was performed as described in Appendix B2. An *Hpa*I + *Xba*I double digest was used to confirm the size of the plasmid containing the RT gene to confirm genetic stability of the plasmids. A *Xho*I digest was used to confirm the pCONEPI harbouring plasmids.

5.2.7. STATISTICAL ANALYSES

IFN- γ ELISPOT responses to an individual peptide induced by each of the rBCG Δ panCD vaccines were statistically analyzed using Student's *t* test for unpaired data and *p* values of <0.05 were considered significant.

5.3. RESULTS

5.3.1. IMMUNOGENICITY OF THE rBCG HIV-1 SUBTYPE C VACCINE VECTOR EXPRESSING THE RT ANTIGEN

Immune responses of mice to the vaccines, rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] were studied since these vaccines displayed good vector stability and consistent expression of HIV-1 antigens (Chapter 4). rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] were cultured *in vitro* by growing in the presence of ATc for 1 week to ensure the HIV-1 RT antigen expression was repressed. The cultures were then washed and half were grown in the presence of ATc ($\Delta panCD$ [pPM3RT]+ and rBCG $\Delta panCD$ [pRC3RT]+) and half in the absence of ATc ($\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT]). Vaccine stocks were prepared when the cultures reached mid to late log growth.

5.3.1.1. Assessment of the cellular immune responses in spleens

BALB/c mice were used to examine the rBCG $\Delta panCD$ immunogenicity, since these animals carry the BCG-s (sensitive) allele that makes them sensitive to BCG infection^{93, 99}. The mice were primed with rBCG $\Delta panCD$ via the intraperitoneal route. A dose of 1×10^7 cfu was used, as previous studies carried out in our laboratory where doses of 1×10^3 , 1×10^5 , 1×10^7 and 1×10^8 cfu were compared (unpublished data) indicated that a dose of 1×10^7 cfu BCG induced the highest number of HIV antigen-specific T cells. The rBCG vaccine vector replicates slowly and this site of immunization is well tolerated as it allows BCG replication and stimulation of the immune response without causing any abscess. Mice were boosted 28 days later via the intramuscular route with SAAVI MVA-C²⁹⁰.

The frequency of antigen specific RT-CD8, RT-CD4 and V3-CTL CD8 cells induced in mice after vaccination were evaluated 12 days after the SAAVI MVA-C boost using an IFN- γ ELISPOT assay. Peptide IFN- γ ELISPOT responses to a rBCG $\Delta panCD$ vaccine prime and SAAVI MVA-C boost of > 1.5 fold that of the peptide response to the control rBCG $\Delta panCD$ [pCONEPI]-prime and SAAVI MVA-C-boost regimen were considered positive responses and occurring as a result of the boost. Additionally, the responses in the absence of peptide (R10 medium only) and an irrelevant peptide served as background responses for the assay and were less than 7 ± 4 sfu/ 10^6 splenocytes and were subtracted from the response to the stimulant.

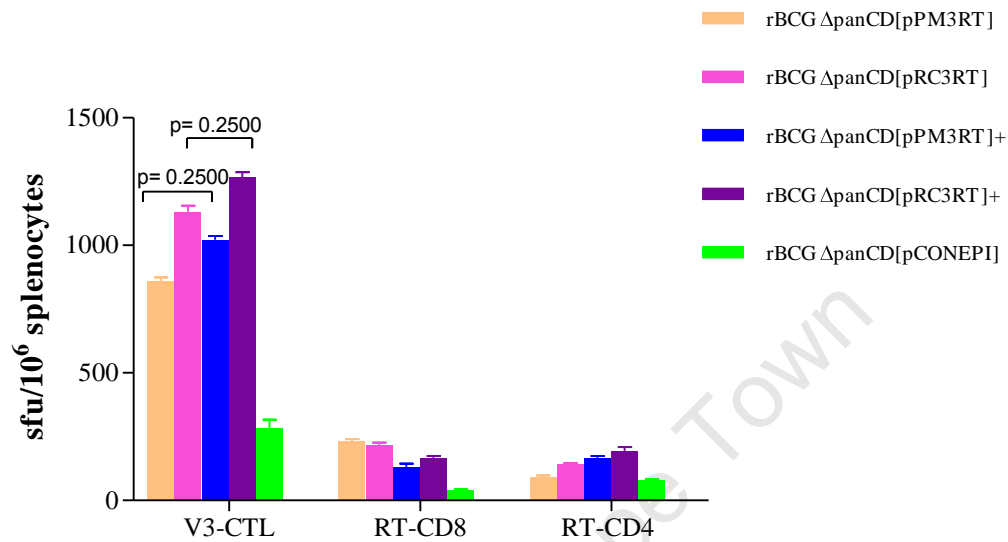
As can be seen in Figure 5.2 panel IFN- γ ELISPOT responses were induced in vaccinated mice. The responses to the three peptides varied in magnitude. A prime with the rBCG $\Delta panCD[pPM3RT]$ vaccine and a boost with SAAVI MVA-C yielded an average response of 858 ± 14 sfu/ 10^6 splenocytes to the V3-CTL CD8 peptide which was 3-fold above the response induced by a rBCG $\Delta panCD[pCONEPI]$ prime and SAAVI MVA-C boost. A prime with the rBCG $\Delta panCD[pRC3RT]$ vaccine and a boost with SAAVI MVA-C yielded an average response of 1183 ± 20 sfu/ 10^6 splenocytes to the V3-CTL CD8 peptide which was 4.2-fold higher than that induced by the rBCG $\Delta panCD[pCONEPI]$ prime and SAAVI MVA-C boost. RT-CD8 peptide responses induced by a prime with rBCG $\Delta panCD[pPM3RT]$ and rBCG $\Delta panCD[pRC3RT]$ and SAAVI MVA-C boost ranged from 132 to 232 ± 10 sfu/ 10^6 splenocytes, which was 3.3 - 5.6-fold above the responses induced by a rBCG $\Delta panCD[pCONEPI]$ prime and SAAVI MVA-C boost. Responses to the RT-CD4 peptide ranged from 90 to 193 ± 10 sfu/ 10^6 splenocytes, which was 1.1 - 2.4-fold above the responses induced by a rBCG $\Delta panCD[pCONEPI]$ prime and SAAVI MVA-C boost. These responses to the RT-CD8 and RT-CD4 peptides were weaker than the responses to the V3-CTL peptide suggesting these RT epitopes were subdominant to the V3-CTL epitope. Thus when the cumulative response to the HIV peptides is considered, rBCG $\Delta panCD[pPM3RT]$ and rBCG $\Delta panCD[pRC3RT]$ can be concluded to be immunogenic in that they primed the immune system to a boost with SAAVI MVA-C.

The effect of growing the vaccines in the presence of ATc and not removing it prior to vaccination on immune response was evaluated. As shown in Figure 5.2 panel A, the responses elicited by the rBCG $\Delta panCD[pPM3RT]^+$ and rBCG $\Delta panCD[pRC3RT]^+$ to HIV-1 peptides were not different from those induced by rBCG $\Delta panCD[pPM3RT]$ and rBCG $\Delta panCD[pRC3RT]$, $p = 0.2500$ (value for the V3-CTL peptide). Additionally, there were no significant differences between the responses induced to the RT peptide ($p > 0.05$). Thus removing ATc from the vaccines prior to vaccination did not alter the immune response significantly.

The immunogenicity of the rBCG HIV-1 subtype C vaccine experiment was repeated to assess reproducibility of the results. However, in this experiment only the rBCG $\Delta panCD[pPM3RT]$ and rBCG $\Delta panCD[pRC3RT]$ vaccines were tested. The results in Figure 5.2 panel B are the average of two independent experiments. The same trends of immune responses were obtained

as those shown in Figure 5.2 panel A, as both vaccines displayed good priming immunogenicity and the V3-CTL CD8 epitope induced the strongest responses.

A.



B.

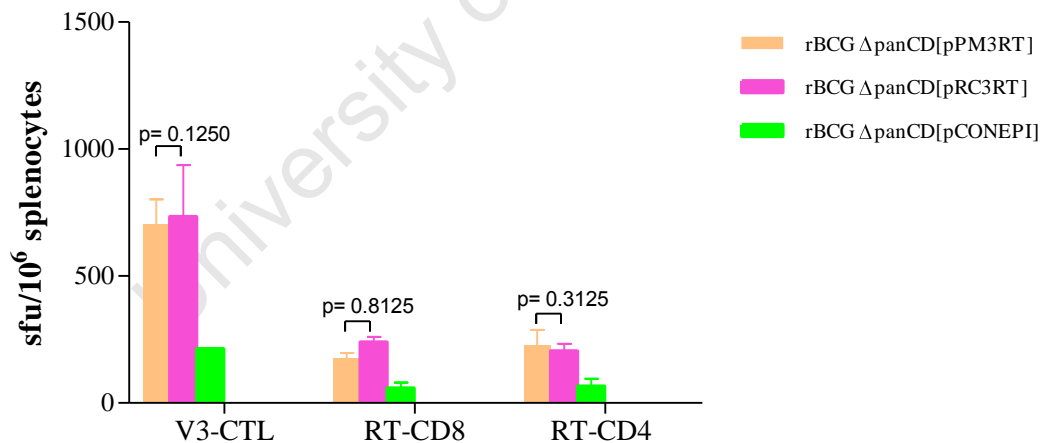


Figure 5.2: IFN- γ ELISPOT responses induced by the rBCG HIV-1 subtype C vaccines grown in absence and presence (+) of ATc. The IFN- γ ELISPOT responses were determined on day 40 in mice after priming with the rBCG HIV-1 RT vaccines (10^7 cfu) on day 0 and boosting with SAAVI MVA-C (10^4 pfu) on day 28. A. Bars represent the mean response of triplicate reactions to the indicated peptides and the standard deviation of the mean response, for a single experiment. B. Bars represent the mean responses from 2 independent experiments. For both A and B, IFN- γ ELISPOT responses to an individual peptide induced by each of the rBCG Δ panCD vaccines were statistically analyzed using the Student's *t* test for unpaired data and *p* values were found to be > 0.05 , indicating thus that there is no significant difference in the responses to the peptides induced by the individual vaccines.

The magnitude of the responses stimulated by the rBCG $\Delta panCD$ vaccines harbouring the *tetRr1.7* mutant was compared to those stimulated by the rBCG $\Delta panCD$ vaccines harbouring *tetRr1.12* mutant. As can be seen in Figure 5.2 the V3-CTL CD8 responses induced by *tetRr1.7* harbouring rBCG $\Delta panCD$ vaccines were approximately 1.3-fold stronger than those induced by the *tetRr1.12* harbouring rBCG $\Delta panCD$ vaccines. However these responses are not significantly different as $p = 0.1250$ (panel B).

The V3-CTL and RT IFN- γ ELISPOT responses induced by a rBCG $\Delta panCD$ [pCONEPI] prime and SAAVI MVA-C boost were lower than those induced by SAAVI MVA-C alone (Figure 5.3). When mice were primed with the control BCG $\Delta panCD$ and boosted with SAAVI MVA-C the response to the V3-CTL peptide was 2.2-fold lower, to the RT-CD8 peptide 5.0-fold lower and to the RT-CD4 peptide 2.5-fold lower than the responses observed with only a SAAVI MVA-C vaccination and no rBCG $\Delta panCD$ vaccine prime. This indicates that BCG infection leads to suppression of immune responses to SAAVI MVA-C.

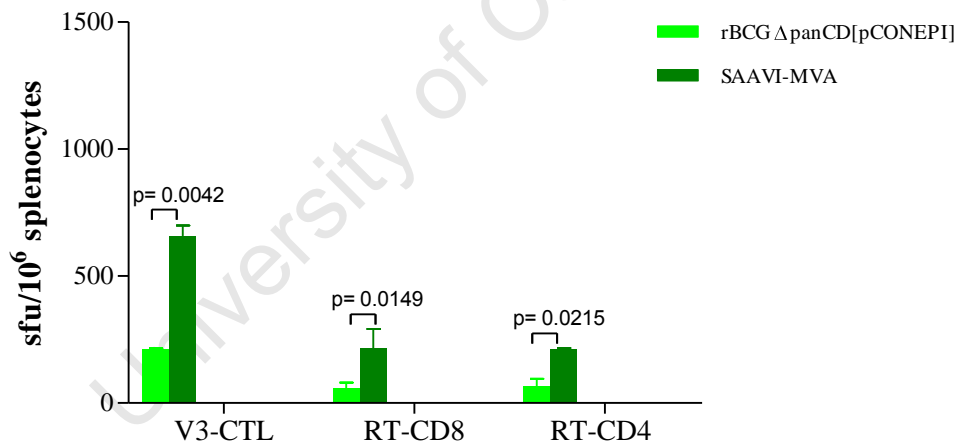


Figure 5.3: IFN- γ ELISPOT responses induced by the rBCG $\Delta panCD$ [pCONEPI] prime plus SAAVI MVA-C boost and responses induced by SAAVI MVA-C alone. The IFN- γ ELISPOT responses were determined on day 40 in mice after priming with the rBCG $\Delta panCD$ [pCONEPI] vaccine (10^7 cfu) on day 0 and boosting with SAAVI MVA-C (10^4 pfu) on day 28. The group of the mice that was vaccinated with SAAVI MVA-C only was vaccinated on day 28. Bars represent the mean responses from 3 independent experiments. Statistical analysis was done using the Student's t test for unpaired data and p values were found to be > 0.05 , indicating that there were significant differences in the responses to the peptides induced by the different vaccine combinations.

In the IFN- γ ELISPOT assay the non-specific polyclonal stimulant ConA was used as a positive control. Splenocytes from all groups of vaccinated mice responded to ConA to a level of 800-1000 sfu/10⁶ splenocytes (data not shown). These results fit with similar data available in the Immunology laboratory (University of Cape Town). Responses induced to the BCG vector were monitored using a lysate of BCG as the stimulant. All the rBCG $\Delta panCD$ vaccines used to prime the immune system induced BCG responses to a level of 450 - 560 sfu/10⁶ splenocytes (Figure 5.4). As can be seen in the Figure, there were no significant differences between the responses induced by rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] in the presence or absence of ATc.

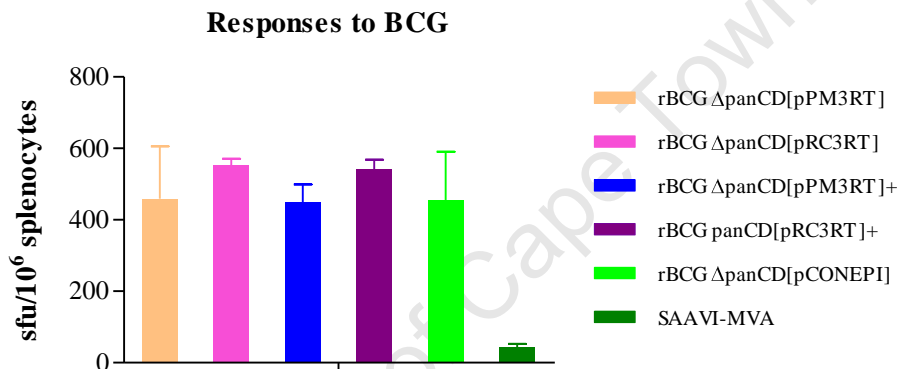
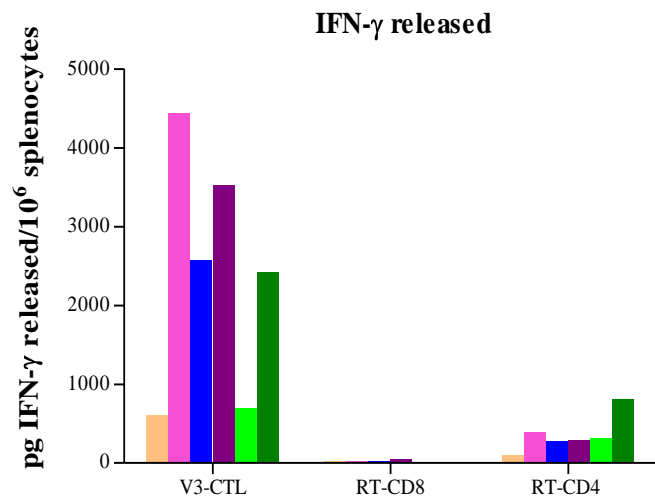


Figure 5.4: IFN- γ ELISPOT assay responses induced on day 40 after a prime with the indicated rBCG $\Delta panCD$ vaccines (10^7 cfu, i.p.) on day 0 and boost with SAAVI-MVA C (10^4 pfu; i.p.) on day 28. Bars represent the mean response of triplicate reactions to the indicated peptides and the standard deviation of the mean response, for a single experiment.

5.3.1.2. Assessment of the cytokines released by the splenocytes

The CBA assay was used to quantify a spectrum of cytokines released into the culture supernatant during stimulation with RT-CD4, RT-CD8 and V3-CTL CD8 peptides. Figure 5.5 shows data for the four different rBCG $\Delta panCD$ vaccinations and SAAVI MVA-C boost. The V3-CTL CD8 cells produced predominantly IFN- γ following priming with rBCG $\Delta panCD$ [pRC3RT], rBCG $\Delta panCD$ [pPM3RT]+ or rBCG $\Delta panCD$ [pRC3RT]+ vaccines and boosting with MVA as the level was 1.5 times greater than that of a prime with the control rBCG $\Delta panCD$ [pCONEPI] and SAAVI MVA-C boost (panel A). TNF- α was also produced predominantly from the V3-CTL CD8 specific cells and was positive for the following vaccine regimens, the prime with rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] and SAAVI

A.



B.

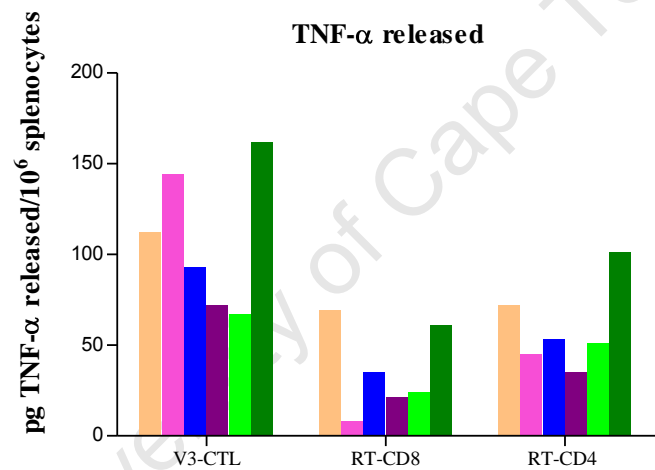


Figure 5.5: Cytokines measured (pg cytokine released / 10^6 splenocytes) in the supernatant harvested from splenocyte cultures that have been stimulated for 48 h with the indicated HIV-1 peptides. Mice were primed with the rBCG $\Delta panCD$ (colour coded) vaccines and then boosted on day 28 with SAAVI MVA-C. Dark green group received SAAVI MVA-C only on day 28. Splenocytes prepared on day 40 from a pool of spleens for each group (5 mice per group) were stimulated in culture with the indicated peptide. Culture supernatants were collected 48 h later. The cytokines released in the supernatant were determined using a CBA assay.

MVA-C boost as the level was 1.5 times greater than that of a prime with the control rBCG $\Delta panCD[pCONEPI]$ and SAAVI MVA-C boost (panel B). No inflammatory HIV specific IL-6, MPC-1, IL-10, and IL-12p70 was detected in the culture supernatant from any of the rBCG $\Delta panCD$ vaccine prime SAAVI MVA-C boost regimens.

Splenocytes from mice vaccinated with SAAVI MVA-C only produced IFN- γ and TNF- α when stimulated with the V3-CTL CD8 and RT-CD4 peptide and TNF- α only when stimulated with the RT-CD8 peptide (Figure 5.5 panel A). No IL-6, IL-10, MCP-1 or IL-12p70 was detected (data not shown). ConA was used to stimulate the cells non-specifically as a positive control for cytokine release. ConA stimulation of splenocytes for all mouse groups produced IFN- γ , TNF- α , IL-6, IL-10, MCP-1 in the culture supernatant. IL-12p70 was not produced since this cytokine is produced by dendritic cells that are not sensitive to ConA (data not shown).

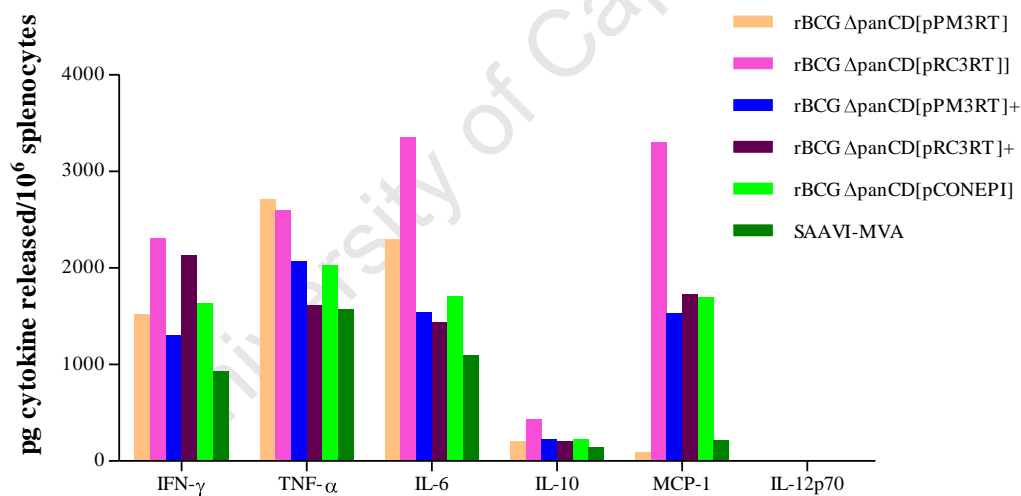


Figure 5.6: Cytokines measured (pg cytokine released /10⁶ splenocytes) in the supernatant harvested from the 48 h BCG stimulated splenocyte cultures. Mice were primed with the rBCG $\Delta panCD$ vaccines (colour coded) and then boosted on day 28 with SAAVI MVA-C. Dark green group received SAAVI MVA-C only on day 28. Splenocytes prepared on day 40 from a pool of spleens for each group (5 mice per group) were stimulated in culture with BCG lysate. Culture supernatants were collected 48 h later. The cytokines released in the supernatant was determined using a CBA assay.

The CBA assay was able to detect IFN- γ , TNF- α , IL-6, IL-10 and MCP-10 in the culture supernatant from splenocytes stimulated with BCG lysate, whereas IL-12p70 could not be detected in the culture supernatant (Figure 5.6). This is thought to be because the BCG lysate is not pure and may contain endotoxins and cross reactive antigens that would account for this response. Splenocytes from mice vaccinated with SAAVI-MVA-C have been shown to release cytokines in response to stimulation with BCG lysate in other experiments carried out by members of our research group too.

5.3.2. SPLENOCYTE NUMBERS AND LYMPHOCYTE PHENOTYPE AFTER VACCINATION

The phenotype of lymphocytes in the spleens was determined by flow cytometry after staining the cells with specific fluorescent antibodies that label CD3 T-cells and the CD4 and CD8 T-cell subsets as well as B-cells. Shown in Table 5.4 are the splenocyte numbers and lymphocyte phenotype. The naïve mice data was obtained from other studies in our Immunology laboratory (University of Cape Town). In comparison to the naïve group, it is clear that the rBCG $\Delta panCD$ vaccination increased the total number of cells in the spleen. This was seen for all groups primed with the rBCG $\Delta panCD$ vaccines. In addition priming with the rBCG $\Delta panCD$ vaccines, rBCG $\Delta panCD$ [pPM3RT] and rBCG $\Delta panCD$ [pRC3RT] did not alter the proportion of CD4 and CD8 T-cells, as indicated by the CD4/CD8 ratio, from that of naïve mice. This was confirmed (data not shown) with splenocytes from 2 further experiments where these vaccines were used (the splenocytes were used in the ELISPOT assay data given in Figure 5.2). A prime with rBCG $\Delta panCD$ [pPM3RT]⁺ and rBCG $\Delta panCD$ [pRC3RT]⁺ appeared to increase the CD4/CD8 T-cell ratio. However these vaccines were not tested further to confirm this change.

5.3.3. CONFIRMATION OF THE GENETIC INTEGRITY OF THE rBCG HIV-1 SUBTYPE C VACCINE VECTORS POST IMMUNIZATION

To verify the stability of the rBCG $\Delta panCD$ vaccines, the splenocytes suspensions isolated from the mouse spleens 40-days post vaccination were diluted appropriately and plated in the presence and absence of antibiotic selection. There was very little or no difference in the number of rBCG $\Delta panCD$ colonies grown in the presence or absence of antibiotic selection, indicating that very little or no plasmid loss from the rBCG $\Delta panCD$ had occurred.

Table 5.4: The percentage of B- and T-cells in the mice spleens of the groups vaccinated with the respective rBCG Δ panCD vaccines (Table 5.1).

VACCINE	NEGATIVE	CD3	CD3/CD4	CD3/CD8	CD4/CD8 RATIO	CD19	CELL/SPLEEN (x10 ⁶)
rBCG Δ panCD[pPM3RT]	11.9	36.1	24.0	11.1	2.2	52.0	118
rBCG Δ panCD[pRC3RT]	13.3	40.9	27.5	12.6	2.2	45.8	83
rBCG Δ panCD[pPM3RT]+	11.8	39.6	31.3	7.7	4.1	48.6	80
rBCG Δ panCD[pRC3RT]+	14.2	42.4	31.1	10.3	3.0	43.4	107
rBCG Δ panCD[pCONEPI]	13.5	38.0	25.5	11.6	2.2	48.5	96
SAAVI MVA-C	18.1	42.6	28.3	13.4	2.1	39.3	46
Naïve mice	15.9	41.4	28.3	12.3	2.3	42.7	45

The splenocytes obtained were stained with CD3-APC, CD4-FITC, CD8-PerCP and CD19-PE fluorescing antibodies and analysed with BD FACS Calibur®. These splenocytes were used in the IFN- γ ELISPOT assay, data shown in Figure 5.2A, and the CBA assay, data shown in Figure 5.4, and assessment of responses to BCG lysate, Figure 5.3 and 5.5.

NEGATIVE: The cells that did not bind to the fluorescing antibodies.

To confirm the genetic integrity of the rBCG $\Delta panCD$ vaccines, plasmid DNA was isolated from rBCG $\Delta panCD$ colonies. The DNA was subjected to restriction enzyme mapping using restriction enzymes. The plasmid DNA isolated from the rBCG HIV-1 subtype C vaccine vectors produced the expected banding patterns after a *XbaI* + *HpaI* double digest, however the bands did not align exactly with the control DNA (Figures 5.7 and 5.8). Consequently plasmid DNA samples from the vaccines were cleaned using the MinElute reaction clean-up kit [Qiagen] and then remapped. The results are shown in Figure 5.9, expected band sizes of 4856 bp and 1644 bp were obtained, which aligned exactly with the control plasmid DNA (two maps from each vaccine are shown). The rBCG $\Delta panCD$ [pCONEPI] appeared to be intact since expected 2259 bp and 1814 bp fragments (produced when the pCONEPI plasmid is digested with *XhoI*) were obtained after restriction digest with this enzyme (Figure 5.10).

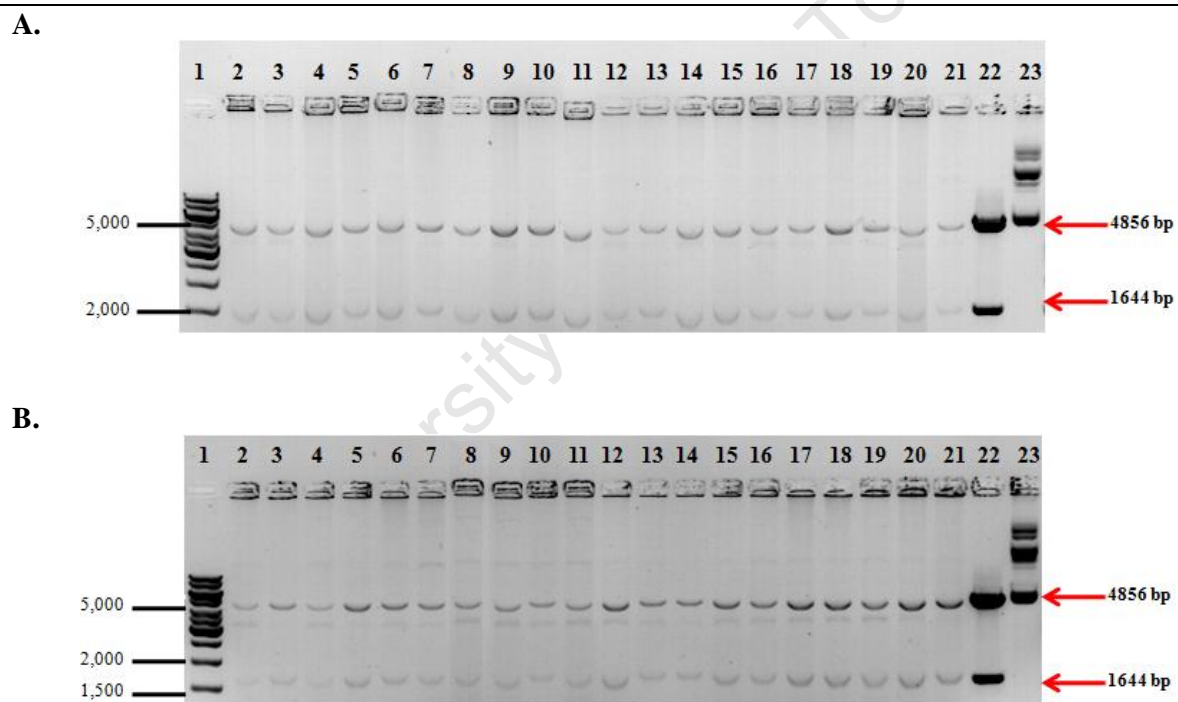
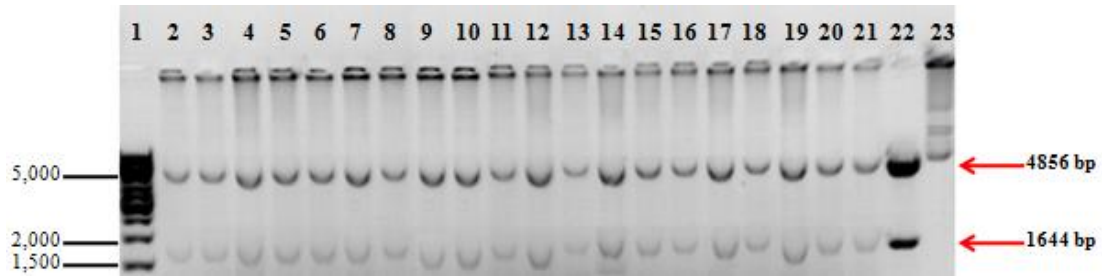


Figure 5.7: Mapping of plasmid DNA extracted from rBCG $\Delta panCD$ [pPM3RT] vaccines isolated from mice splenocyte cultures 40 days post vaccination using *HpaI* and *XbaI*. **A.** rBCG $\Delta panCD$ [pPM3RT]: Lanes, (1) 1 kb ladder [Fermentas]; (2 - 21) plasmid DNA isolated from rBCG $\Delta panCD$ vaccines; (22) purified pPM3RT plasmid DNA; and (23) uncut pPM3RT plasmid DNA. **B.** rBCG $\Delta panCD$ [pPM3RT]⁺: Lanes, (1) 1 kb ladder [Fermentas]; (2 - 21) plasmid DNA isolated from rBCG $\Delta panCD$ vaccines; (22) purified pPM3RT plasmid DNA; and (23) uncut pPM3RT plasmid DNA.

A.



B.

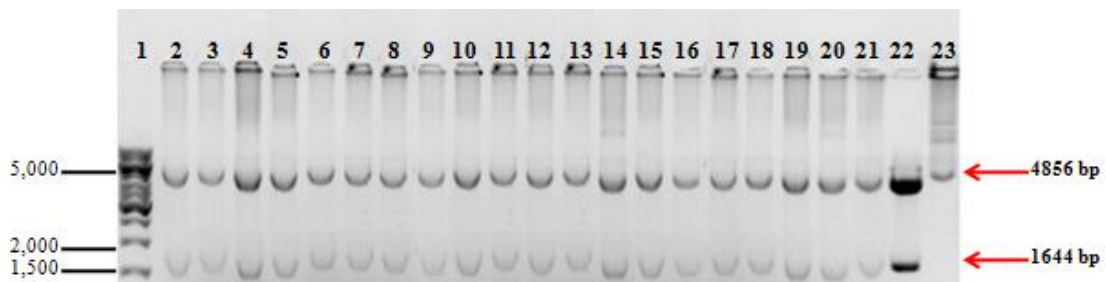


Figure 5.8: Mapping of plasmid DNA extracted from rBCG $\Delta panCD$ [pRC3RT] vaccines isolated from mice splenocyte cultures 40 days post vaccination using *HpaI* and *XbaI*. A. rBCG $\Delta panCD$ [pRC3RT]: Lanes, (1) 1 kb ladder [Fermentas]; (2 - 21) plasmid DNA isolated from rBCG $\Delta panCD$ vaccines; (22) purified pRC3RT plasmid DNA; and (23) uncut pRC3RT plasmid DNA. B. rBCG $\Delta panCD$ [pRC3RT]+: Lanes, (1) 1 kb ladder [Fermentas]; (2 - 21) plasmid DNA isolated from rBCG $\Delta panCD$ vaccines; (22) purified pRC3RT plasmid DNA; and (23) uncut pRC3RT plasmid DNA.

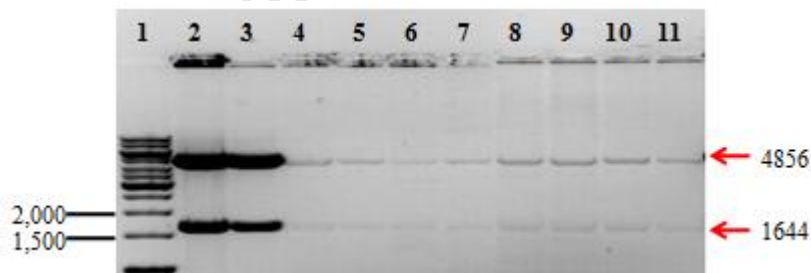


Figure 5.9: Mapping of plasmid DNA extracted from rBCG HIV-1 subtype C vaccines isolated from mice splenocyte cultures 40 days post vaccination using *HpaI* + *XbaI*. Lanes, (1) 1 kb ladder [Fermentas]; (2) purified pPM3RT plasmid DNA; (3) purified pRC3RT plasmid DNA; (4 - 5) plasmid DNA isolated from rBCG $\Delta panCD$ [pPM3RT] vaccines; (6 - 7) plasmid DNA isolated from rBCG $\Delta panCD$ [pRC3RT] vaccines; (8 - 9) plasmid DNA isolated from rBCG $\Delta panCD$ [pPM3RT]+ vaccines and (10 - 11) plasmid DNA isolated from rBCG $\Delta panCD$ [pRC3RT]+ vaccines.

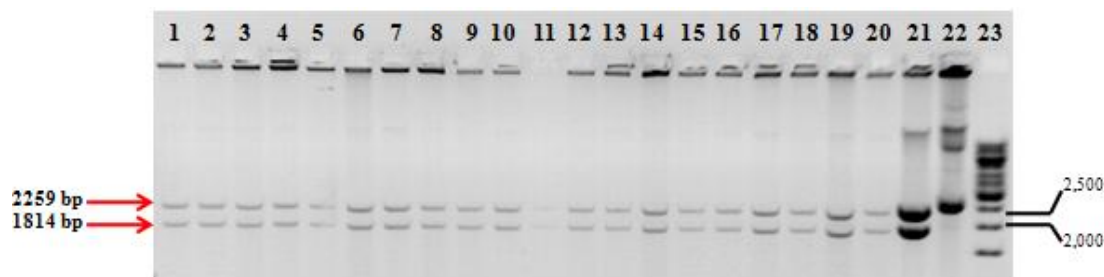


Figure 5.10: Mapping of plasmid DNA extracted from rBCG $\Delta panCD$ [pCONEPI] vaccine isolated from mice splenocyte cultures 40 days post vaccination using the *Xho*I digest. Lanes, (1-20) plasmid DNA isolated from rBCG $\Delta panCD$ [pCONEPI] vaccines; (21) purified pCONEPI plasmid DNA; and (22) uncut pCONEPI plasmid DNA; and (23) 1 kb ladder [Fermentas].

5.4. DISCUSSION

The aim of the study was to evaluate the immunogenicity of the ATc-dependent rBCG $\Delta panCD$ vaccines and was done in BALB/c mice given their common use in various studies for the testing of rBCG vaccines. Both cellular and humoral immune responses have been elicited in these mice to both the bacteria and expressed recombinant antigens^{107, 112, 116, 133, 134}.

Immune responses to the recombinant RT antigen (tagged with a BALB/c V3-CTL CD8 peptide) expressed by the rBCG $\Delta panCD$ vaccines in this study was investigated using a prime-boost vaccine regimen, where the rBCG $\Delta panCD$ vaccines were used to prime the immune system and a recombinant pox virus vaccine expressing a matching immunogen to boost the induced primary immune response. This approach was based on results from several studies, where although modest responses to the recombinant immunogen could be detected when the rBCG vaccine was used on its own, high cellular responses to the recombinant protein was detected after a heterologous booster vaccine was administered^{131, 132, 156, 159}. It was concluded from these studies that rBCG vaccines efficiently prime the immune system and elicit immunogen-specific memory CD8 lymphocytes that expand in response to a boost for a heterologous vaccine. Thus the most efficient way of enhancing an immune response to the recombinant BCG antigen is by priming the immune system with an rBCG vaccine followed by a heterologous boost vaccine.

The ability of rBCG to efficiently prime the immune system to the recombinant gene may be linked to the fact that it is a replicating vaccine. Due to its slow replication ability, BCG replication within the host is expected to be slow occurring over a period of 3 weeks post vaccination and would favour a low level of antigen expression within the bacteria together with

a low level of antigen presentation^{7, 291, 292}. Subsequently a low magnitude of antigen specific T-cells would be induced. We suspect that such cells differentiate very soon after vaccination to the memory phenotype. These memory T cells expand rapidly and differentiate into effector cells that produce cytokines following re-exposure to the antigen via the boost^{293, 294}. Inflammatory signals from the BCG vector may also play a major role in the induction of the recombinant antigen-specific memory cells²⁹⁵. *M. smegmatis* expressing HIV-1 gp120 induced antigen specific helper CD4 T-cells and memory CD8 T-cells²⁹⁵. These memory T-cells rapidly expanded to recombinant adenovirus expressing the same antigen.

Our rBCG HIV-1 subtype C vaccines remained genetically stable during *in vitro* replication of the vaccine vectors and post vaccination, which is important for the induction of the immune responses against the RT antigen that we observed. IFN- γ producing V3-CTL-specific CD8 T-cells as well IFN- γ producing RT- specific CD8 and CD4 T-cells were generated by all the rBCG $\Delta panCD$ vaccines when used as a priming vaccine and followed by a SAAVI MVA-C boost. The responses achieved were above those to the control, rBCG $\Delta panCD$ [pCONEPI] prime and SAAVI MVA-C boost by 3 - 4.2-fold (to the V3-CTL CD8 peptide), 3.3 - 5.6-fold (to the RT-CD8 peptide) and 1.1 - 2.4-fold (to the RT-CD4). Our findings were in accord with data reported from numerous studies using rBCG as a priming vaccine together with a heterologous vaccine as a booster vaccine in mice^{111, 131}. Promkhaykaew *et al.*, (2009)¹⁴⁷ demonstrated induction of specific CTLs against the Gag epitope in BALB/c mice immunized with rBCG/HIV-1gagE (Chapter 1, Table 1.1). However, using the same vaccine they showed increased levels of CTLs that were long lasting where the animals were primed with rBCG/HIV-1gagE and boosted with rVaccinia/HIV-1gagE (Table 1.1). Most recently Rosario *et al.*, (2010)¹³¹ tested the immunogenicity of their novel candidate vaccine, rBCG expressing an HIV-1 clade A-derived immunogen in BALB/c mice and rhesus macaques. On its own the rBCG induced very weak T-cell responses, whereas as a prime followed by a boost with recombinant MVA and ovine adenovirus expressing the same immunogen, the vaccine induced robust HIV-1-specific T-cell responses. Generally the BCG primed heterologous boost regimen increases vaccine take by the host by boosting the pathogen insert-specific responses whilst avoiding the accumulation of anti-vector immunity¹²⁵.

Ideally the vaccines should induce a durable HIV-1-specific cell-mediated immune response as these responses have been associated with the anti-viral effect^{146, 283, 296}. Vaccine specific

polyfunctional CD8⁺ T-cells (CTLs) can be predicted to be beneficial for protection against viral infection based on the role HIV-specific CD8 T-cells play in the control of viral infection in SIV infected non-human primates^{144, 297-299}. Virus-specific CTLs are also considered to control viremia in HIV infected humans^{145, 146, 296}. Virus-specific CTLs mediate the anti-HIV immunity by killing the infected CD4 T-cells¹⁴⁶.

Even though the CD4 T-helper cells are the primary HIV infected cells, uninfected CD4 T-helper cells found during the long asymptomatic phase function to help maintain an effective immunity against the virus³⁰⁰⁻³⁰². Additionally, the role of CD4 T-cells can be both through direct and indirect protection, since HIV specific CTLs decline overtime when virus-specific CD4⁺ T-cells have been depleted. The RV 144 HIV vaccine trial in Thailand in 2009 has indicated that HIV specific CD4 T-cells appear to play an important role in protection³⁰³. Thus we can deduce from our results that the rBCG $\Delta panCD$ vaccines could have the potential to control the viral infection seeing that they were able to induce these responses.

Valor *et al.*, (2008)²⁹⁶ reported that the ability of CD8 T-cells to produce cytokines is one of the main ways in which they display their cytotoxic activity, as the cells producing these cytokines have been associated with cytotoxic activity in HIV-1 infected individuals. Furthermore, cytokines released from HIV-specific cells in response to stimulation with HIV peptides defines the anti-viral effector functions of the vaccine induced cells as well as whether the vaccine induced response is predominantly a Th1 or Th2 response. In the CBA assay, the HIV-1 peptide-specific lymphocytes induced by the rBCG $\Delta panCD$ vaccine prime SAAVI MVA-C boost regimens produced mainly IFN- γ and TNF- α cytokines, and no peptide specific IL-10 was produced (Figure 5.5), indicating that the HIV response was predominantly Th1. These results were in agreement with many rBCG studies that have demonstrated that Th1 cytokines, particularly IFN- γ and TNF- α , are produced in response to the recombinant antigen subsequent to rBCG vaccination^{102, 105-108}. The mechanisms that lead to the induction of predominantly Th1 cytokine production following rBCG immunization were reviewed in Chapter 1.

The immune response is influenced by the quantity of the recombinant antigen presented to the host, which in turn is influenced by the antigen expression levels within the vaccine^{291, 292}. In our system the levels of the antigen expression were influenced by the induction kinetics of the rBCG $\Delta panCD$ vaccines after removal of ATc. In Chapter 4 we demonstrated that the

recombinant antigen levels produced in rBCG $\Delta panCD$ after removing ATc were equivalent to the levels produced in rBCG $\Delta panCD$ containing ATc (Figure 4.9). It is expected that the rBCG $\Delta panCD$ vaccines that contained ATc and those without ATc produced comparable levels of the HIV-1 RT antigen within the mice, and hence it was not surprising the vaccines displayed similar immunogenicity. This also indicates that the vaccines could be produced without having to remove ATc before vaccination of the animals.

The V3-CTL and RT responses induced by a rBCG $\Delta panCD$ [pCONEPI] prime and SAAVI MVA-C boost were lower than those induced by the control SAAVI MVA-C boost only (Figure 5.3). The expression of suppressor of cytokine signalling (SOCS) could account for the reduction in responses to the SAAVI MVA-C boost following a BCG prime. Imai *et al.*, (2003)³⁰⁴ reported that BCG infection induces the production of SOCS in dendritic cells and macrophages as well as SOCS negative regulators of IFN- γ signal transduction, and that this correlates with the suppression of activation of non-receptor tyrosine kinases, Janus kinase 1 and 2 and phosphorylation of the signal transducer and activator of transcription 1 (JAK/STAT) signalling stimulated by IFN- γ and thus results in a poor immune response. On the other hand the responses induced in the SAAVI MVA-C vaccinated mice were not suppressed and consequently were very high.

The data obtained from genetic stability evaluation confirmed that the rBCG $\Delta panCD$ vaccines recovered from the spleens 40-days post vaccination were genetically intact since they retained both the antibiotic resistant gene as well as the HIV-1 *rt* gene.

Overall, this experiment demonstrated that our rBCG HIV-1 subtype C vaccines could remain stable within the host and deliver T cell epitopes to the MHC class I and II molecules for processing and presentation to the CD8 and CD4 cells. Additionally, it was established that the levels of IFN- γ induced after priming with rBCG $\Delta panCD$ [pRC3RT], harbouring the *tetRr1.7* mutant were above those induced after priming with rBCG $\Delta panCD$ [pPM3RT], containing the *tetRr1.12*, however this was not significant. This data indicates that the vaccines harbouring the TetRr1.7 repressor and those harbouring the TetRr1.12 repressor were similar in terms of immunogenicity.

CHAPTER 6: SUMMARY AND CONCLUSION

In this study a TetRr1.12 dependant regulatory system was constructed, which consisted of the regulatory and the responsive components of the TetR system for stringent regulation, i.e. a TetRr1.12 repressor; and a strong *P_{smyc}* mycobacterial promoter engineered to contain *tetO* operator sequences. This system was designed to regulate the expression of the recombinant HIV antigens in *M. bovis* BCG Δ *panCD* in the presence of the co-repressor, ATc. We aimed to apply the regulatory system to vaccine design, such that the recombinant antigen expression is repressed *in vitro* by the presence of ATc to ensure genetic stability during manufacture and then the lack of ATc will result in higher antigen expression *in vivo* in order to elicit strong immune responses. The tetracycline regulated system (harbouring the Tet system) has been employed in vaccine design³⁰⁵, however, the tetracycline inducible system was used thus the vaccinees have to be given tetracycline to activate the expression of the vaccine antigens, which is undesirable. In this current study we employed the reverse tetracycline regulated system in rBCG Δ *panCD* expressing GFP or HIV-1 antigens, which is novel. In this system recombinant antigen expression occurs in the absence of tetracycline, so vaccinees do not have to be given tetracycline.

By assessing the TetRr1.12 system in *M. smegmatis* and BCG Δ *panCD* using GFP as a model antigen, we confirmed that the levels of GFP expressed could be modified by varying the amount of ATc. The minimum ATc concentration required to repress GFP expression was determined. The induction of GFP expression upon ATc removal was poor in *M. smegmatis* none of the recombinants showed maximal induction, whereas rBCG Δ *panCD* showed complete induction, This data indicates that the TetRr1.12 system would be ideal for repression and gene silencing studies, as Ehrt *et al.*, 2005, Kamionka *et al.*, 2005 and Gandotra *et al.*, 2007^{200, 222, 255} have shown using the TetRr1.7 repressor.

The stability assessment of the recombinants showed that *in vitro* regulation of the recombinant antigen expression improved genetic stability when GFP and HIV-1 RT were used as the antigens. However, the recombinants expressing the Gag antigen were not stable. Increasing the concentration of ATc in the cultures during vaccine preparation to 3.0 μ g/ml would have completely repressed Gag expression, which may have improved stability of the recombinants. However, following removal of ATc (at 3.0 μ g/ml) induction of recombinant expression was

slow and did not reach maximal levels. This revTetR regulatory system will need further optimisation to be used as a rBCG-based AIDS vaccine to ensure stable rBCG vaccines expressing HIV-1 Gag and envelope can be generated.

Klotzsche *et al.*, (2009)¹⁹⁸ have constructed two new revTetRs, TetR#27 and TetR#28 that repress the expression of the target gene more efficiently than the TetRr1.7 repressor in the presence of ATc. These repressors may suppress expression of HIV-Gag sufficiently to allow the preparation of stable vaccine stocks. However, Klotzsche *et al.*, (2009)¹⁹⁸ did not evaluate the induction of the expression of the target gene upon removal of ATc. Thus maximal levels of the target gene induction following ATc removal may also be difficult to achieve using the TetR#27 and TetR#28 repressors, as we have found using the TetRr1.7 and TetRr1.12 repressors.

Having tested the TetRr1.12 system we established that proper functioning lies in the combination of the intensity of repression in the presence of ATc and subsequent induction upon its removal, which is influenced by the following factors:

- The ATc concentration.
- The amount of TetRr1.12 repressor produced, which depends on the strength of the promoter responsible for its expression (Guo *et al.*, 2007)¹⁹⁷.
- Affinity of the repressor for the *tetO* operators in the presence of ATc, since it influences the release of the TetRr1.12 repressor from the *tetO* operators upon ATc removal.
- The orientation of insertion of the TetRr1.12 expression cassette relative to the recombinant antigen, as placing the cassette in the same orientation as the recombinant antigen improved the stability of the systems.

Investigation of the immunogenicity of rBCG $\Delta panCD$ vaccines expressing RT in BALB/c mice showed that the vaccines were immunogenic, as antigen-specific intracellular IFN- γ expression was induced in mice splenocytes in response to stimulation with HIV-1 peptides following a prime with rBCG $\Delta panCD$ vaccines and a boost with SAAVI MVA-C. These results indicate that we overcame the challenges of low antigen expression caused by genetic instability that can lead to the weak immunogenicity usually associated with expression of viral antigens in BCG. As the rBCG $\Delta panCD$ strain shows limited replication *in vivo* it may be suitable for administration to HIV-infected individuals. Development of a vaccine against both tuberculosis and HIV would be highly beneficial in the developing world. Thus future studies using this system could include the development of a dual HIV/TB vaccine.

APPENDICES

APPENDIX A: STRAINS AND CULTURE TECHNIQUES

A1 Culturing conditions of *E. coli*

E. coli DH5 α strain from our laboratory stocks was used for plasmid DNA replication. *E. coli* DH5 α was grown on 2YT agar (Appendix C) at 37°C and propagated in 2YT broth (Appendix C) at 37°C on shakers for aeration. Recombinant *E. coli* clones were grown in 2YT broth supplemented with 25 μ g/ml Kan [Sigma-Aldrich]. For plasmid selection, cells were plated on 2YT agar supplemented with 25 μ g/ml Kan and incubated overnight at 37°C.

A2 *E. coli* competent cells preparation

E. coli cells were made competent using a modification of the dimethyl sulphoxide (DMSO) method by Chung and Miller in 1988³⁰⁶. A single colony of *E. coli* was picked off a freshly streaked 2YT agar plate, used to inoculate 5 ml of 2YT broth without antibiotic selection and incubated overnight at 37°C. The culture was diluted 1:100 in 100 ml of 2YT broth and propagated for approximately 3 hrs to early log phase (OD₆₀₀ 0.2 - 0.4). The cells were harvested by centrifugation at 5000 rpm for 5 min at 4°C. The pellet was resuspended in 1:10 volume (10 ml) of ice-cold sterile TSB buffer (Appendix C). The cells were placed on ice for 10 min and either transformed with plasmids immediately or stored in sterile glycerol to a final concentration of 10% v/v in 100 μ l aliquots at -80°C.

A3 Transformation of plasmid DNA into *E. coli*

The standard cold-shock transformation methodology was used for transformation into *E. coli* cells³⁰⁷. The glycerol stock aliquots of competent cells (100 μ l) were thawed on ice. Plasmid DNA (0.1 - 100 ng) was mixed with the cells and placed on ice immediately and incubated for 20 min. The cells were heat-shocked at 37°C for 5 min. A volume of 1 ml 2YT broth pre-warmed to room temperature was added. The vials were incubated at 37°C for 60 min to allow for expression of the antibiotic resistance marker. During this incubation step, the tubes were gently mixed by inversion three to four times. Aliquots of 50 μ l, 100 μ l and 200 μ l were plated onto the appropriate selective 2YT solid media. The remaining transformation mix was pelleted, resuspended in 100 μ l 2YT broth and plated as well.

A4 Preparation of *E. coli* electro-competent cells

A single colony of *E. coli* was picked off a freshly streaked LB agar plate, used to inoculate 5 ml of LB broth (Appendix C) and incubated overnight at 37°C. The culture was diluted 1:1000 in 100 ml of LB broth pre-warmed to 37°C and propagated at 37°C. At mid-log phase (OD₆₀₀ = 0.8) the culture was divided into 2 in 50 ml sterile tubes and centrifuged at 4000 rpm at 4°C for 20 min. The supernatants

were discarded. The cells were washed twice by, resuspending the pellets in 50 ml of 10% glycerol, centrifuging at 4000 rpm at 4°C for 20 min and discarding the supernatants. After washing, each pellet was resuspended in 25 ml of 10% glycerol. The cells were then pooled into 50 ml sterile tube, centrifuged at 4000 rpm at 4°C for 20 min and resuspended in 2 ml of 10% glycerol. The cell suspension was aliquoted in volumes of 100 µl in 1.5 ml sterile eppendorf tubes [Merck] and stored at -80°C.

A5 Electroporation into *E. coli*

The Gene Pulser™ [Bio-Rad] set at Resistance: 200 ohms, Voltage: 2.5 kV, Capacitance: 125 µFD was used to electroporate the cells. The glycerol stock aliquots of *E. coli* electro-competent cells (100 µl) were thawed on ice. Plasmid DNA (5 µl) was mixed with the cells and made up to a final volume of 50 µl in 10% glycerol, transferred to a pre-chilled 0.1 mm gap cuvette [Bio-Rad] and then electroporated. A volume of 500 µl of SOC media (Appendix C) was added after electroporation, and the mixture was incubate at 37°C for 1 hr. Aliquots of 100 µl and 200 µl of the transformation cultures were plated on LB Agar (Appendix C) containing Kan (25 µg/ml) and incubated at 37°C overnight.

APPENDIX B: STANDARD PROTOCOLS

B1 Plasmid isolation

B1.1 Small-scale plasmid isolation procedure

Small-scale plasmid DNA isolations were performed using the alkaline lysis method ³⁰⁸. Single *E. coli* bacterial colonies were used to inoculate 800 µl 2YT medium containing Kan (25 µg/ml) in 1.5 ml eppendorf tubes [Merck] and grown overnight at 37°C. The bacteria containing tubes were centrifuged at 14 000 rpm for 2 min at room temperature in the centrifuge 5417C [Eppendorf]. The supernatant was discarded and the remaining cells were resuspended in 200 µl solution I (Appendix C) and incubated at room temperature for 10 min. A volume of 400 µl of solution II (Appendix C) was added to each tube of the resuspended cells and the tubes were placed on ice for 10 min. A volume of 300 µl of solution III (Appendix C) was added to each tube and mixed gently. The tubes were then placed on ice for 10 min in order to precipitate cell debris, protein and chromosomal DNA. The mixture was centrifuged at 14 000 rpm for 5 min at room temperature. A volume of 900 µl of each supernatant was transferred to a new eppendorf tube, to which 600 µl of isopropanol [Merck] was added and mixed. The tubes were left at room temperature for 2 min to allow the plasmid DNA to precipitate. The samples were then centrifuged at 14 000 rpm for 15 min at room temperature to pellet plasmid DNA. The supernatants were discarded and the remaining pellets were washed in 0.5 ml ice cold 70% ethanol and then air dried for 5 min. The pellets were dissolved in 50 µl TE buffer [Appendix C] and stored at -20°C until further required.

B1.2 Large-scale plasmid isolation procedure

Plasmid DNA was isolated using the Genopure plasmid midi kit [Roche] for low-copy plasmids. A single bacterial colony of *E. coli* containing the appropriate plasmid was inoculated into 100 ml 2YT broth containing Kan (25 µg/ml). The inoculated culture was grown overnight at 37°C, shaking vigorously. From the overnight culture, 700 µl was removed and added to 300 µl of 50% (v/v) glycerol for storage at -80°C in a 1.5 ml sterile eppendorf tube. The remaining culture was harvested by centrifugation at 4 000 rpm in the centrifuge 5810R [Eppendorf] for 15 min at 4°C. The rest of the procedure was done according to manufacturer's instructions. For maximum plasmid recovery, subsequent to the elution step, 5 ml of eluent was divided into five 800 µl aliquots in 2 ml eppendorf tubes [Merck], followed by the addition of 700 µl of isopropanol to each tube to precipitate plasmid DNA and then centrifugation at 15000 rpm for 15 min at 4°C. Supernatant was removed, each pellet washed with 500 µl of 70% ethanol and the tubes were then centrifuged at 15000 rpm for 15 min at 4°C. The supernatants were removed and the pellets were air dried for 10 min. Finally the pellets were resuspended in 50 µl HPLC grade H₂O, pooled and quantified using a Nanodrop Spectrophotometer [Inqaba Biotech].

B2 Restriction digests

The purified plasmid DNA was restricted with restriction endonucleases in a 1.5 ml eppendorf tube to a final volume of 20 µl unless otherwise indicated. For a single digest, the reaction contained: 1 µl (10 U) enzyme, 1X reaction buffer, 500 ng DNA and H₂O to make it up to 20 µl. For a double digest, the reaction contained: 1 µl (10 U) of each enzyme, 1X reaction buffer, 500 ng DNA and H₂O to make it up to 20 µl. The contents were mixed by pulse spinning and incubated in a water bath at 37°C (unless otherwise specified) for 2 hrs. Digested products were analyzed on a 1% agarose gel (Appendix B3).

B3 Agarose gel electrophoresis

Following any purification procedure, the integrity and concentration of DNA was confirmed on a 1% (w/v) mini agarose slide gel (10 ml) prepared using electrophoresis grade agarose [Sigma], dissolved in Tris-borate-EDTA (TBE) buffer (Appendix C) by heating and after cooling to ≈55°C, 0.5 µg/ml ethidium bromide (EtBr) (Appendix C) was added and the gel was allowed to solidify. The mini gel was electrophoresed at 10 V for 15 min. Restriction digests were analysed on 1% standard agarose gels (150 ml) for 1.5 hrs at 90 V or on maxi agarose gels (250 ml) for 1.5 hrs at 140 V using the *Pst*I [Roche] digested λDNA [Roche] as a marker or 1 kb size marker [Promega]. All the agarose gels were visualised over a UV box and digital images captured and edited with Uvipro [Uvitech Silver] equipment and software.

B4 Cloning of DNA

The desired DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit [Qiagen] according to manufacturer's instructions. When necessary, to prevent spontaneous religation of the plasmid backbone, dephosphorylation reactions were performed with the Shrimp Alkaline Phosphatase (TSAP) [Roche] to remove 5' phosphate groups and a T4 DNA polymerase kit [Roche] was used for filling in 5' overhangs with unlabelled dNTPs. Ligation reactions were performed using a T4 DNA ligase kit [Roche] according to manufacturer's instructions in 1.5 ml sterile eppendorf tubes and incubated overnight at room temperature. The vector: insert ratio used was 1:3, using plasmid DNA at 50 ng. Plasmid DNA was purified from reaction mixes using a QIAquick Purification Kit [Qiagen] according to manufacturer's instructions.

B5 Protein extraction

B5.1 Bead-beating/Fastprep method of cell free lysate preparation

Proteins were extracted from mycobacterial cultures propagated at 37°C. The cultures were centrifuged at 10 000 rpm (25 ml per tube) for 10 min at 4°C, and the pellets were resuspended in 5 ml of ice cold sterile PBS pH 7.2 [Gibco].

The samples were transferred into FastRNA tubes [Bio 101] containing 0.1 mm zirconia-silica beads [BioSpec] that had been sterilised by autoclaving, 1 ml culture per 0.5 g beads. The cells were then lysed using the FastPrep machine [Bio 101 MP Biomedicals]. The samples were processed for 30 seconds at speed 6 and then placed on ice until cool. This regime was repeated three times. The samples were then centrifuged at 14000 rpm at room temperature for 10 min. The supernatants were retained and centrifuged a second time to remove residual debris. The supernatants (cell lysates) were retained, divided into three aliquots and stored at -20°C for use as a control for ELISPOT. One of these was quantified as in Appendix B6.

B5.2 SDS boiling method

Cultures of the selected recombinant mycobacterial colonies were prepared and grown to a desired OD. A volume of 20 ml from each culture was centrifuged at 4 000 rpm at room temperature for 5 min in the Centrifuge 5417C [Eppendorf]. The pellets were resuspended in 20 ml of PBS, centrifuged as before and resuspended in 250 µl SDS lysis buffer (Appendix C). Samples were then boiled for 30 min and then allowed to cool to room temperature before being centrifuged at 14 000 rpm for 5 min. The supernatants were aliquoted into fresh 1.5 ml eppendorf tubes and stored at -80°C. Protein concentration of all samples was determined (Appendix B6).

B6 Protein concentration determination using Bio-Rad DC protein assay

The concentration of protein lysates was quantified using the standard Dc-Bio-Rad protein quantification assay kit [Bio-Rad] according to manufacturer's instructions, which utilises the Bovine Serum Albumin (BSA) as a standard [Roche]. The DC protein assay is for protein concentration determination following detergent solubilisation and the reaction is based on the Lowry assay³⁰⁹. Several dilutions of the BSA standard were prepared ranging from 0.1 to 2.0 µg/µl in SDS lysis buffer. The protein samples were diluted 10 fold in SDS lysis buffer.

Protein lysates and BSA standards were dispensed into a 96-well flatbottom MicroWell™ plate [Greiner Bio-one GmbH], 5µl sample per well and each sample was loaded in triplicate. A volume of 25 µl Reagent A' (Appendix C) was added to each well followed by the addition of 200 µl Reagent B. SDS lysis buffer was used as a blank to ensure that it did not affect OD readings. The mixtures were vortexed and then incubated at room temperature for 15 min. The absorbances were measured at 750 nm using the VERSAmax microplate reader [Molecular Devices]. The microplate reader subtracted the blank values and plotted the standard curve using the BSA standard absorbance values. The standard curve was then used to determine the unknown protein concentrations by extrapolation using the absorbance values of the protein samples.

B7 Protein Assessment Procedures

Fluorimetry assay and Western blotting were used to assess comparative levels of protein expression resulting from the recombinant mycobacteria. Fluorimetry was used to assay GFP levels and SDS-PAGE + Western blotting were used to determine HIV antigen expression levels.

B7.1 Fluorimetry assay

The expression of GFP was assessed with the use of the whole-cell GFP Fluorescence assay described by Carroll and Parish, 2004. This method detects fluorescence from the samples and thus in our case it measured the amount of GFP activity of the recombinant constructs. Recombinant mycobacterial cultures (20 - 30 ml) from the mid-logarithmic growth phase (OD = 0.5) were centrifuged at 4 000 rpm for 15 min in 50 ml tubes [Coring]. The pellets were resuspended in PBS to OD₆₀₀ of 2 units/100µl. The samples were vortexed briefly and then loaded into a 96-well white Fluorimetry plate [NUNC], 100 µl of sample per well and each sample was loaded in triplicate. PBS was loaded as a blank to ensure that it did not affect fluorescence readings. *rM. smegmatis*[pCONEPI], was used as a negative control to account for background interference caused by mycobacterial proteins. Fluorescence readings were taken using a Varian Cary Eclipse Fluorimeter [Seco Mann] set to read GFP fluorescence at an excitation wavelength of 485 nm and emission wavelength of 515 nm.

To correct for any inaccuracies encountered during the concentration process, the following calculation was done: OD₆₀₀ readings of the sample pellets resuspended in PBS were taken to determine the correction factor (2OD units/ OD₆₀₀ reading). Fluorimetry results were calculated by taking an average of the triplicate readings, multiplying it by the correction factor, subtracting the average of the *rM. smegmatis*[pCONEPI] readings, and then expressing GFP expression as relative fluorescence units (rfu).

B7.2 HIV Antigen expression analysis

B7.2.1 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were isolated from recombinant mycobacterial cultures using the SDS boiling method (Appendix B5.2). Each protein sample (60 µg) was mixed with (20%) Sample buffer (Appendix C). The samples were boiled for 5 min, cooled in ice and analyzed on 12.5% Criterion XT Tris acetate denaturing gel [Bio-Rad]. Kaleidoscope Molecular Weight Marker [Bio-Rad] was used as a standard. Purified RT and Gag proteins from our Laboratory were used as positive controls and cell lysate from rBCG Δ *panCD*[pCONEPI] was used as a negative control. Initially the SDS-PAGE was run at 100 V for 20 min, and later increased to 150 V for 55 min using 1X Tricine Running Buffer [Bio-rad].

B7.2.2 Western Blotting Analysis

Prior to protein transfer, the gels, the extra thick filter papers and the PVDF membranes were pre-treated in separate plastic dishes. The gels and the extra thick filter papers were equilibrated in 20 ml Transfer buffer (Appendix C) in small open containers for a maximum period of 30 min. The PVDF membranes were soaked in 20 ml 100% methanol for 2 min, the methanol was poured off and the membranes were then rinsed in distilled H₂O with intermittent shaking. After 5 min, the water was discarded and 20 ml protein transfer buffer (Appendix C) was added to the membranes and left for 20 min. A TransBlot semi-dry transfer cell [BioRad] set at 25 V for 60 min was used to transfer proteins.

The membranes were briefly washed with room temperature TBS (Appendix C), and incubated in Ponceau S (Appendix C) for 2 min to check for proteins on the membrane and finally rinsed with distilled H₂O. Subsequently the membranes were incubated in Block/Wash buffer (Appendix C) on a shaker for 2 hrs and then probed with the primary antibody diluted in Block/Wash buffer (1:5000) overnight at 4°C, slightly shaking. The membranes were washed 4 times in Block/Wash buffer (4 x 15 min) at room temperature after which they were hybridised with the secondary antibody diluted 1:10 000 in Block/Wash buffer and incubated for 1 hr at room temperature. The membranes were then washed 4 times in Block/Wash buffer (4 x 15 min) and then developed using Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) [Roche].

APPENDIX C: LIST OF MEDIA AND SOLUTIONS

1. MEDIA

2YT agar: 16 g tryptone powder; 10 g yeast extract; 5 g NaCl; 15 g agar per litre.

2YT broth: 16 g tryptone powder; 10 g yeast extract; 5 g NaCl per litre.

LB Agar: 10 g tryptone; 5 g yeast extract; 5 g NaCl₂; 12 g bacto agar per litre.

LB broth: 10 g tryptone; 5 g yeast extract; 5 g NaCl₂ per litre.

MB-7H10 agar: 19 g MB-7H9 agar [Difco]; 100 ml OADC [Biolabs]; 0.63% w/v glycerol) per litre.

MB-7H9 broth: 4.7 g MB-7H9 broth [Difco]; 100 ml OADC [Biolabs]; 0.025% v/v tyloxapol [Sigma-Aldrich]; 0.25% w/v glycerol per litre.

R10: 88.9% RPMI 1640 with Glutamax [Gibco] + 10% FBS + 1% Pen/Strep; 50 µM 2-ME.

RPMI complete: RPMI + 10% v/v FCS; 1% Pen/Strep; 10 µM/ml fungin; 50 µM 2-ME.

SOC: 20 g tryptone; 5 g yeast extract; 0.5 g NaCl₂; 1.86 g; KCl; 5 ml 1M MgCl₂; 20 ml 1M glucose per litre.

2. SOLUTIONS

ATc: ATc powder [BDH] dissolved in HPLC H₂O.

Blocking: 1% rat serum; 1% mouse serum; CD16/CD32 mix, antibody mix in FACS buffer.

EtBr: EtBr powder [BDH] dissolved in distilled H₂O.

GTE-lysozyme: 20 mg/ml lysozyme powder [Sigma] dissolved in GTE buffer.

Kan: kanamycin powder [Sigma-Aldrich] dissolved in HPLC H₂O.

Nova Red Substrate [Sothorn Cross]: 3 drops of Reagent 1; 2 drops Reagent 2; 2 drops Reagent 3 and 2 drops H₂O₂ to 15 ml H₂O.

Pen-Strep: 10,000 units (g/ml) Penicillin; 10,000 µg/ml Streptomycin in normal saline.

Ponceau S stain: 0.1% w/v ponceau S; 5% v/v acetic acid.

Reagent A': 1:50 reagent S [Bio-Rad] in reagent A [Bio-Rad].

Solution I: 1 M Tris-Cl pH 8.0; 20% w/v glucose; 0.5 M EDTA pH 8.0.

Solution II: 10% SDS; 10 M NaOH.

Solution III: 11.5% v/v glacial acetic acid; 5 M potassium acetate pH 4.8 per 100 ml.

Turks: 0.02% w/v gentian/crystal violet; 7% v/v glacial acetic acid per 100 ml.

3. BUFFERS

Block /Wash: 1 X TBS; 0.5 % v/v Tween-20; 4 % w/v elite milk powder.

Coating: 1x PBS pH 7.2 [Gibco].

Dilution: 1x PBS + 10% FBS.

FACS: PBS containing 1% FCS and 0.1% NaN₃.

Lysis: 5 mM Tris-HCL; 140 mM NH₄Cl, pH 7.3.

Protein transfer: 0.303% Tris; 1.44% glycine.

SDS lysis: 3% w/v SDS; 10% v/v glycerol; 62.5 mM Tris-HCl, pH 6.8.

Sample: 0.5 M Tris-HCL, pH 6.8; 2% SDS, 5% β- mercaptoethanol, 15% Glycerol; 1% bromophenol blue.

RBC lysis: 0.15 M NH₄Cl. 10 mM KHCO₃, 0.1 mM EDTA and distilled H₂O [Sigma-Aldrich, SA]

Resuspension: 8.5% w/v NaCl; 10% glycerol; 10% tyloxapol.

TBE: 10.8 g Tris; 5.5 g boric acid; 0.5 M EDTA, pH 8.0 per litre.

TBS: 50 mM Tris; 150 mM NaCl, pH to 7.5.

TE: 10 mM Tris-Cl, pH 7.5; 1 mM EDTA.

Tricine running: 20x Tricine running buffer [Biorad] diluted to 1X in distilled H₂O.

TSB: 16 g peptone powder; 11 g yeast extract powder; 5 g NaCl; 10% w/v PEG, 5% v/v DMSO, 10 mM MgCl₂, 10 mM MgSO₄ per litre.

TSBG: 20% w/v glucose; 100 ml TSB.

Wash buffer I: 1x PBS - 0.05% Tween 20, pH 7.4 [Sigma].

Wash buffer II: 1x PBS pH 7.4 [Sigma].

APPENDIX D: SEQUENCES

D1 TetRr1.7 protein showing the following amino acid mutations in the protein sequence: E to A at position 15; L to G at position 17; L to V at position 25

```

                                     M _ S _ R _ L _ D _ K _ S _ K _
V _ I _ N _ S _ A _ L _ A _ L _ G _ N _ E _ V _ G _ I _ E _ G _ V _ T _ T _ R _
K _ L _ A _ Q _ K _ L _ G _ V _ E _ Q _ P _ T _ L _ Y _ W _ H _ V _ K _ N _ K _
R _ A _ L _ L _ D _ A _ L _ A _ V _ E _ I _ L _ A _ R _ H _ H _ D _ Y _ S _ L _
P _ A _ A _ G _ E _ S _ W _ Q _ S _ F _ L _ R _ N _ N _ A _ M _ S _ F _ R _ R _
A _ L _ L _ R _ Y _ R _ D _ G _ A _ K _ V _ H _ L _ G _ T _ R _ P _ D _ E _ K _
Q _ Y _ D _ T _ V _ E _ T _ Q _ L _ R _ F _ M _ T _ E _ N _ G _ F _ S _ L _ R _
D _ G _ L _ Y _ A _ I _ S _ A _ V _ S _ H _ F _ T _ L _ G _ A _ V _ L _ E _ Q _
Q _ E _ H _ T _ A _ A _ L _ T _ D _ R _ P _ A _ A _ P _ D _ E _ N _ L _ P _ P _
L _ L _ R _ E _ A _ L _ Q _ I _ M _ D _ S _ D _ D _ G _ E _ Q _ A _ F _ L _ H _
G _ L _ E _ S _ L _ I _ R _ G _ F _ E _ V _ Q _ L _ T _ A _ L _ L _ Q _ I _ V _
```

D2 **TetRr1.12** protein showing the following amino acid mutations in the protein sequence: V to G at position 20; G to R at position 21; I to N at position 22

```

M S R L D K S K V I N
S A L E L L N E G R N E G L T T R K L A
Q K L G V E Q P T L Y W H V K N K R A L
L D A L A V E I L A R H H D Y S L P A A
G E S W Q S F L R N N A M S F R R A L L
R Y R D G A K V H L G T R P D E K Q Y D
T V E T Q L R F M T E N G F S L R D G L
Y A I S A V S H F T L G A V L E Q Q E H
T A A L T D R P A A P D E N L P P L L R
E A L Q I M D S D D G E Q A F L H G L E
S L I R G F E V Q L T A L L Q I V *

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Table D3.1: Description of the P_{smyc} promoter-operator sequences on the parental plasmids (modified Mayat, (2009)²⁶³).

PLASMID NAME	SEQUENCE LENGTH (BP)	SEQUENCE	FEATURES
pNM3	199	5' -TCTAGAGGATCCTGTGCGTTTCGCACGCACAGGC CCGGTGTGAGAAGGGTCTCTGCAGAGCGGGAGAACT <u>CCCTATCAGTGATAGAGT</u> TTGTCC <u>TCCCTATCAGTG</u> <u>ATAGATA</u> GGCTCTGGGA G TACCCGTGTGTACGACCA GCACGGCATAACATCATTTTCGACGCCGAGAGATTTCG CGCCCGAAATGAGCACGAATTC-3'	Full-length P_{smyc} promoter with two $tetO_2$ sequences situated on either side of -10
pNM4	200	5' -TCTAGAGGATCCTGTGCGTTTCGCACGCACAGGC CCGGTGTGAGAAGGGTCTCTGCAGAGCGGGAGAACA <u>CTCTATCATTGATAGAGT</u> TTGTCC <u>ACTCTATCATTG</u> <u>ATAGAGT</u> AGGCTCTGGGA G TACCCGTGTGTACGACC AGCACGGCATAACATCATTTTCGACGCCGAGAGATTTCG CGCCCGAAATGAGCACGAATTC-3'	Full-length P_{smyc} promoter with two $tetO_1$ sequences situated on either side of -10

Transcription start sites (+1) are boxed.

Blue sequences represent $tetO_2$ and those in

Red sequences represent $tetO_1$

Underlined 6bp sequences represent -35 and -10 regions

Table D3.2: Description of the episomal TetRr1.7 dependant regulatory system designs. These plasmids were constructed by inserting the TetRr1.7 cassette into the pNM3 and pNM4 vector backbones.

	PLASMID NAME	FEATURES
TetRr1.7 plasmids	pNM3.1	pNM3 containing the TetRr1.7 cassette in the same orientation as the <i>gfp</i> gene
	pNM3.2	pNM3 containing the TetRr1.7 cassette in the opposite orientation to the <i>gfp</i> gene
	pNM4.1	pNM4 containing the TetRr1.7 cassette in the same orientation as the <i>gfp</i> gene
	pNM4.2	pNM4 containing the TetRr1.7 cassette in the opposite orientation to the <i>gfp</i> gene

D4 pPM3Gag sequence: Showing the following fragments highlighted in:

- TetRr1.12 cassette: pink,
- *P_{myc}* promoter: blue with the operators underlined,
- HIV-1 *gag* gene yellow,
- V3-SV5 epitope: grey and

The deleted fragment underlined>.

```

1      GCTAGCAACAAAGCGACGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAA
61     ATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTA
121    TGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACATGGATG
181    CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCCGGCAATCAGGTGCGACAATCT
241    ATCGCTTGTATGGGAAGCCCCATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCG
301    TTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAAATTTATGCCTC
361    TTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCCTGCGA
421    TCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTG
481    TTGATGCGCTGGCAGTGTTCCTCGCCCGGTTGCATTTCGATTCTGTTTGTAAATGTCCTT
541    TTAACAGCGATCGCGTATTTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGG
601    TTGATGCGAGTGATTTTGTATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAG
661    AAATGCATAATCTTTTGCCATTCTCACCAGGATTCAGTCGTCCTCATGGTGATTTCTCAC
721    TTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCG
781    GAATCGCAGACCGATAACCAGGATCTTGCCATCCTATGGAAGTGCCTCGGTGAGTTTCTC
841    CTTCAATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAAT
901    TGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATGGTTGTAAC
961    ACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTT
1021   TGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGCAAA
1081   GCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTC
1141   CCTCACTTCTGGCTGGATGATGGGGCGATTGAGGCTGGTATGAGTCAGCAACACCTTC
1201   TTCACGAGGCAGACCTCACTAGTTCCTGAGCGTCAGACCCCGTAGAAAAGATCAAAGG
1261   ATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAAACAAAAAACACC
1321   GCTACCAGCGGTGGTTTTGTTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAC
1381   TGGCTTCAGCAGAGCGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCA
1441   CCACTTCAAGAACTCTGTAGCACCAGCTACATACCTCGCTCTGCTAATCCTGTTACCAGT
1501   GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGTTGGACTCAAGACGATAGTTACC
1561   GGATAAGGCGCAGCGGTGCGGGCTGAACGGGGGTTTCGTGCACACAGCCCAGCTTGGAGCG
1621   AACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAAGCGCCACGCTTCC

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1681 CGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCAC
 1741 GAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA GTCTGTGCGGTTCGCCACCT
 1801 CTGACTTGAGCGTCGATTTTTTGTGATGCTCGTCAGGGGGCGGAGCCTATGAAAAACGC
 1861 CAGCAACGCGGCCTTTTTACGGTTCTGGCCTTTTTGCTGGCCTTTTTGCTCACATGTTCTT
 1921 TCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC
 1981 CGCTCGCCCGAGCCGAACGACCAGCGCAACCGGTGAGCCCACCAGCTCCGTAAGTTCGG
 2041 GTGCTGTGTGGCTCGTACCCGCGCATTCAGGCGGCAGGGGGTCTAACGGGTCTAAGGCGG
 2101 CGTGTACGGCCGCCACAGCGGCTCTTAGCGGCCCGAAACGTCTCGAAACGACGCATGT
 2161 GTTCTCTCTGGTTGGTACAGGTGGTTGGGGGTGCTCGGCTGTGCTGGTGTTCATCATC
 2221 AGGGCTCGACGGGAGAGCGGGGAGTGTGAGTTGTGGGGTGGCCCTCAGCGAAAATATC
 2281 TGACTTGGAGCTCGTGTGCGACCATAACCGGTGATTAATCGTGGTATTATATCAAGCGT
 2341 GAGCCACGTCGCGGACGAATTTGAGCAGCTCTGGCTGCCGTAAGTCCCTGGCAAGCGA
 2401 CGATCTGCTCGAGGGGATCTACCGCCAAAGCCGCGCGTTCGGCCCTAGGCCGCCGGTACAT
 2461 CGAGGCGAACCCAACAGCGCTGGCAAACCTGCTGGTTCGTGGACGTAGACCATCCAGACGC
 2521 AGCGCTCCGAGCGCTCAGCGCCCCGGGGTCCCATCCGCTGCCAACCGCATCGTGGGCAA
 2581 TCGCGCCAACGGCCACGCACACGAGTGTGGGCACTCAACGCCCTGTTCCACGCACCGA
 2641 ATACGCGCGGCGTAAGCCGCTCGCATAACATGGCGGCGTGCGCCGAAGGCCTTCGGCGCGC
 2701 CGTCGATGGCGACCGCAGTTACTCAGGCCTCATGACCAAAAAACCCCGCCACATCGCCTG
 2761 GGAAACGGAATGGCTCCACTCAGATCTCTACACACTCAGCCACATCGAGGCCGAGCTCGG
 2821 CGCGAACATGCCACCGCCGCGCTGGCGTCAGCAGACCACGTACAAAGCGGCTCCGACGCC
 2881 GCTAGGGCGGAATTGCGCACTGTTCCGATTCCGTCAGGTTGTGGGCTATCTTCCCGCCCT
 2941 CATGCGGATCTACCTGCCGACCCGGAACGTGGACGGACTCGGCCGCGCGATCTATGCCGA
 3001 GTGCCACGCGCGAAACGCCGAATTTCCGTGCAACGACGTGTGTCCCGGACCGCTACCCGA
 3061 CAGCGAGGTCCGCGCCATCGCCAACAGCATTGCGGTTGGATCACAACCAAGTTCGCGCAT
 3121 TTGGGCGGACGGGATCGTGGTCTACGAGGCCACACTCAGTGCAGCGCCATGCGGCCATCTC
 3181 GCGGAAGGGCGCAGCAGCGCGCACGGCGGCGAGCACAGTTGCGCGGCGCGCAAAGTCCGC
 3241 GTCAGCCATGGAGGCATTGCTATGAGCGACGGCTACAGCGACGGCTACAGCGACGGCTAC
 3301 AACTGGCAGCCGACTGTCCGCAAAAAGCGGCGGCTGACCGCCGCGGAAGGCGCTCGAATC
 3361 ACCGGACTATCCGAACGCCACGTGTCGCGGCTCGTGGCGCAGGAACGCAGCGAGTGGTTC
 3421 GCCGAGCAGGCTGCACGCCCGAACGCATCCGCGCCTATCACGACGACGAGGGCCACTCT
 3481 TGGCCGAAACGGCCAAACATTTCCGGCTGCATCTGGACACCGTTAAGCGACTCGGCTAT
 3541 CGGGCGAGGAAAGAGCGTGCGGCAGAACAGGAAGCGGCTCAAAAAGGCCACAACGAAGCC
 3601 GACAATCCACCGCTGTTCTAACGCAATTTGGGAGCGGGTGTGCGGGGGTTCGTTGGGGG
 3661 GTTCCGTTGCAACGGGTTCGGACAGGTAAAAGTCCCTGGTAGACGCTAGTTTTCTGGTTTG
 3721 GCCATGCCTGTCTGTTGCGTGTTCGTTGCGTCCGTTTTGAATACCAGCCAGACGAGAC
 3781 GGGGTTCTACGAATCTTGGTGCATACCAAGCCATTTCCGCTGAATATCGTGGAGCTCACC
 3841 GCCAGAATCGGTGGTTGTGGTGTGATGTACGTGGCGAACTCCGTTGTAGTGCTTGTGGTGGC
 3901 ATCCGTGGCGCGGCCGCGGTACCAGATCTTTAAAT **GGGCGAATTGGGGCGGCCACTAGT**
 3961 **CTGCAGCAAGGTCGAACGAGGGGCATGACCCGGTGCGGGGCTTCTTGCACTCGGCATAGG**

EcoRV

4021 **CGAGTGCTAAGAATAACGTTGGCACTCGATATCTGACACTTGAGGAGGAGAGCAAAAATTC**
 4081 **TCATGTGCGCCTGGACAAGTCAAGGTCATCAACTCGGCCCTGGAGCTGCTGAACGAGG**
 4141 **GCCGCAACGAGGGCCTGACCACCCGCAAGCTGGCCAGAAGCTGGGCGTCGAGCAGCCGA**
 4201 **CCCTGTACTGGCACGTGAAGAACAAGCGCGCCCTGCTGGACGCCCTGGCCGTGGAGATCC**
 4261 **TGGCCCGCCACCACGACTACTCGCTGCCGGCCGAGGCGAGTTCGTGGCAGTTCGTTCTGCG**
 4321 **GCAACAACGCCATGTCGTTCCGCCGCGCACTGCTGCGCTACCGCGACGGCGGAAGGTGC**
 4381 **ACCTGGGCACCCGCCCGGACGAGAAGCAGTACGACACCGTGGAGACCCAACTGCGCTTCA**
 4441 **TGACCGAGAACGGCTTCTCGCTGCGCGACGGCCTGTACGCCATCTCGGCCGTGTCGCACT**
 4501 **TCACCCTGGGCGCGGTGCTGGAGCAGCAGGAGCACACCGCCGCCCTGACCGACCGCCCGG**
 4561 **CAGCCCCGGACGAGAACCTGCCGCCGCTGCTGCGGGAGGCCCTGCAAATCATGGACTCGG**
 4621 **ACGACGGCGAGCAGGCGTTCTGCACGGCCTGGAGTCCGCTGATCCGCGGCTTCGAGGTGC**

4681 AACTGACCGCCCTGCTGCAAATCGTGTGAGGGCCCCGAGCGAGGAGCCCGGTCCCTTTGTG

XbaI

4741 GGGCCGGGCTCCTCTGGTTGGTACTCTAGATTAATTAATCCAGCTTTTGTTCCTCTACA
4801 GAGCGGGAGAACTCCCTATCAGTGATAGAGTTGTCTCCCTATCAGTGATAGATAGGCT
4861 CTGGGAGTACCCGTGTGTACGACCAGCACGGCATAACATCATTTTCGACGCCGAGAGATTCG

EcoRI

4921 CCGCCCGAAATGAGCACGAATTC TGACACTTGAGGAGGAGAGCAAAATT CTCATGGGTTA
4981 CGTAATCGATATGGGCGCGCGCCTCGATCCTGCGCGGCGAGAAGCTGGACAAGTGGGA
5041 AAAGATCCGCTGCGCCCCGGGCGGCAAAAAGCACTACATGCTGAAGCACATCGTGTGGGC
5101 CTCGCGCGAGCTGGAACGCTTCGCCCTGAACCCCGGCTGCTGGAAACCTCGGAAGGCTG
5161 CAAGCAGATCATGAAGCAGTTGCAGCCGGCCTTGCAGACCGGCACCGAAGAGCTGAAGTC
5221 GCTGTACAACACCGTGGCCACCCTGTACTGCGTGCACGAGAAGATCGAAGTGCAGCACAC
5281 CAAGGAAGCCCTGGACAAGATCGAAGAAGAACAAGTCCAGCAGAAAGACCCAGCA
5341 GGCCAAGGCCGCCGACGGCAAGGTGTGCGAGAAGTACGGATCCCCGATCGTGCAGAACCT
5401 GCAAGGCCAGATGGTGCACCAGGCCATCTCGCCGCGCACCCCTGAACGCTGGGTGAAGGT
5461 GATCGAAGAAAAGGCCTTCTCGCCGGAAGTGATCCCGATGTTTCACCGCCCTGTTCGGAAGG
5521 CGCGACCCCGCAGGACCTGAACACCATGCTGAACACCGTGGGCGGCCACCAGGCCCAT
5581 GCAGATGCTGAAGGACACCATCAACGAAGAAGCGGCCGAATGGGACCGCCTGCACCCGT
5641 GCACGCCGGCCCGATCGCCCCGGGCCAGATGCGCGAGCCCCGCGGCAGCGACATCGCCGG
5701 CACCACCTCGACCCTGCAAGAACAGATCGCCTGGATGACCTCGAACCCCCCGATCCCCGT
5761 GGGCGACATCTACAAGCGCTGGATCATCCTGGGCCTGAACAAGATCGTGCAGCATGTACTC
5821 GCCGGTGTGATCCTGGACATCCGCCAGGGTCCGAAGGAACCGTTCCGCGACTACGTGGA
5881 CCGCTTCTTCAAGACCCTGCGCGCCGAGCAGGCCACCCAAGAGGTGAAGAAGTGGATGAC
5941 CGACACCCTGCTGGTGCAGAACGCCAACCCCGACTGCAAGACCATCCTGCGCGCCCTGGG
6001 TCCCGGCGCGACCCCTGGAAGAGATGATGACCGCCTGCCAGGGCGTGGGCGGCCCGGGCA
6061 CAAGGCCCGCGTGTGCTGAATATTGCCGAAGCCATGTGCGAGACCAACTCGGGCAACATCAT
6121 GATGCGAGCGCTCGAACTTCAAGGGTCCGCGCCGCATCGTGAAGTGTCTCAACTGCGGCAA
6181 GGAAGGCCACATCGCCCCGCAACTGCCCGGCCCGCGCAAGAAAGGCTGCTGGAAGTGTGG
6241 CAAGGAAGGTCATCAGATGAAGGACTGCACCGAGCGCCAGGCCAACTTCTGGCAAGAT
6301 CTGGCCGTGCGACAAGGGCCGCCCGGGCAACTTCTGCAAAACCGCCGGAACCGACCGC
6361 CCCGCCGGCCGAGTGTTCGCTTCGAAGAGACCACCCCGGCCCGAAGCAGGAACCGAT
6421 CGAGCGCAACCGCTGACCTCGCTGAAGAGCCTGTTCGGCTCGGACCCGCTGTGCGAGGA

EcoRV

6481 TATCGGCGCCCCGCTGCGCGGCCCGGCCGCGCCTTCGTGACCATCACCTTCAAGCAGAT

HpaI

6541 CCCGAACCCCTGCTGGGCTGGACCAGGGTTAACTAGCGTACGATCGACTGCCAGGCAT
6601 CAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTGTTTGTCC
6661 GGCCATCATGGCCGCGGTGATCA

D5 pRC3Gag sequence: Showing the following fragments highlighted in::

TetRr1.7 cassette: red,

P_{smc} promoter: blue with the operators underlined,

HIV-1 *gag* gene: yellow,

V3-SV5 epitope: grey and

The deleted fragment underlined.

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1      GCTAGCAACAAAGCGACGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAA
61     ATATATCATCATGAACAATAAAAAGTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTA
121    TGAGCCATATTCAACGGGAAACGTCCTTGCTCGAGGCCGCGATTAAATTTCCAACATGGATG
181    CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCT
241    ATCGCTTGTATGGGAAGCCCCATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCG
301    TTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAAATTTATGCCTC
361    TTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCCTGCGA
421    TCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTTCAGGTGAAAATATTG
481    TTGATGCGCTGGCAGTGTTCCCTGCGCCGGTTGCATTTCGATTCTGTGTTGTAATTGTCCTT
541    TTAACAGCGATCGCGTATTTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTG
601    TTGATGCGAGTGATTTTGTATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAG
661    AAATGCATAATCTTTTGCCATTCTACCGGATTTCAGTCGTCACTCATGGTGATTTCTCAC
721    TTGATAACCTTATTTTTGACGAGGGGAAAATTAATAGGTTGTATTGATGTTGGACGAGTCG
781    GAATCGCAGACCGATAACCAGGATCTTGCCATCCTATGGAAGTGCCTCGGTGAGTTTTCTC
841    CTTCAATTACAGAAACGGCTTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAAT
901    TGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAAC
961    ACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTT
1021   TGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGCAAA
1081   GCAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTC
1141   CCTCACTTTCTGGCTGGATGATGGGGCGATTACGGCCTGGTATGAGTCAGCAACACCTTC
1201   TTCACGAGGCAGACCTCACTAGTTCCTACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGG
1261   ATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACC
1321   GCTACCAGCGGTGGTTTTGTTTGCOGGATCAAGAGCTACCAACTTTTTTCCGAAGGTAAC
1381   TGGCTTCAGCAGAGCGCAGATACCAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCA
1441   CCACTTCAAGAACTCTGTAGCACCAGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGT
1501   GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACC
1561   GGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCG
1621   AACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAAAGCGCCACGCTTCC
1681   CGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTTCGGAACAGGAGAGCGCAC
1741   GAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCCGGGTTTCGCCACCT
1801   CTGACTTGAGCGTCGATTTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGC
1861   CAGCAACCGCGCCTTTTTTACGGTTCCCTGGCCTTTTTGCTGGCCTTTTTGCTCACATGTTCTT
1921   TCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC
1981   CGCTCGCCGAGCCGAACGACCGAGCGCAACCGGTGAGCCCACCAGCTCCGTAAGTTCGG
2041   GTGCTGTGTGGCTCGTACCCGCGCATTACGGCGCAGGGGTCTAACGGGTCTAAGGCGG
2101   CGTGTACGGCCGCCACAGCGGCTCTTAGCGGCCCGAAACGTCCTCGAAACGACGCATGT
2161   GTTCCCTCCTGGTTGGTACAGGTGGTTGGGGGTGCTCGGCTGTGCTGGTGTTCATCATC
2221   AGGGCTCGACGGGAGAGCGGGGAGTGTGCAGTTGTGGGGTGGCCCCCTCAGCGAAATATC
2281   TGACTTGGAGCTCGTGTGCGACCATAACCGGTGATTAATCGTGGTTTATTATCAAGCGT
2341   GAGCCACGTGCGCCGACGAATTTGAGCAGCTCTGGCTGCCGTACTGGTCCCTGGCAAGCGA
2401   CGATCTGCTCGAGGGGATCTACCGCCAAAGCCGCGGTGCGCCCTAGGCCGCCGGTACAT
2461   CGAGGCGAACCCAAACAGCGCTGGCAAACCTGCTGGTTCGTGGACGTAGACCATCCAGACGC
2521   AGCGCTCCGAGCGCTCAGCGCCCCGGGGTCCCATCCGCTGCCCAACGCGATCGTGGGGCAA
2581   TCGCGCCAACGGCCACGCACACGCAGTGTGGGCACTCAACGCCCTGTTCCACGCACCGA
2641   ATACGCGCGGCGTAAGCCGCTCGCATAACATGGCGGCGTGCGCCGAAGCCTTCGGCGCGC
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2701 CGTCGATGGCGACCGCAGTTACTCAGGCCTCATGACCAAAAACCCCGGCCACATCGCCTG
2761 GGAAACGGAATGGCTCCACTCAGATCTCTACACACTCAGCCACATCGAGGCCGAGCTCGG
2821 CGCGAACATGCCACCGCCGCGCTGGCGTCAGCAGACCAGTACAAAGCGGCTCCGACGCC
2881 GCTAGGGCGGAATTGCGCACTGTTTCGATTCCGTCAGGTTGTGGGCTATCTTCCCGCCCT
2941 CATGCGGATCTACCTGCCGACCCGGAACGTGGACGGACTCGGCCGCGGATCTATGCCGA
3001 GTGCCACGCGCGAAACGCCGAATTTCCGTGCAACGACGTGTGTCCCGGACCGCTACCGGA
3061 CAGCGAGGTCCGCGCCATCGCCAACAGCATTGCGGTTGGATCACAACCAAGTCGCGCAT
3121 TTGGGCGGACGGGATCGTGGTCTACGAGGCCACACTCAGTGC GCGCCATGCGGCCATCTC
3181 GCGGAAGGGCGCAGCAGCGCGCACGGCGGCGAGCACAGTTGCGCGGCGCGCAAAGTCCGC
3241 GTCAGCCATGGAGGCATTGCTATGAGCGACGGCTACAGCGACGGCTACAGCGACGGCTAC
3301 AACTGGCAGCCGACTGTCCGCAAAAAGCGGCGCGTGACCGCCGCCGAAGGCGCTCGAATC
3361 ACCGGACTATCCGAACGCCACGTCGTCCGGCTCGTGGCGCAGGAACGCAGCGAGTGGTTC
3421 GCCGAGCAGGCTGCACGCCGCGAACGCATCCGCGCCTATCAGGACGACGAGGGCCACTCT
3481 TGGCCGCAAACGGCCAAACATTTCCGGGCTGCATCTGGACACCGTTAAGCGACTCGGCTAT
3541 CGGGCGAGGAAAGAGCGTGC GGCAGAACAGGAAGCGGCTCAAAGGCCACAACGAAGCC
3601 GACAATCCACCGCTGTTCTAACGCAATTGGGGAGCGGGTGTGCGGGGGTTCCGTGGGGG
3661 GTTCCGTTGCAACGGGTCGGACAGGTAAAAGTCCCTGGTAGACGCTAGTTTCTGGTTTGG
3721 GCCATGCCTGTCTCGTTGCGTGTTCGTTGCGTCCGTTTTGAATACCAGCCAGACGAGAC
3781 GGGTTCTACGAATCTTGGTCGATACCAAGCCATTTCCGCTGAATATCGTGGAGCTCACC
3841 GCCAGAATCGGTGGTTGTGGTGATGTACGTGGCGAACTCCGTTGTAGTGCTTGTGGTGGC
3901 ATCCGTGGCGCGGCCGCGGTACCAGATCTTTAAAT **CTAGTCTGCAGCAAGGTCGAACGAG**
3961 **GGGCATGACCCGGTGC GGGGCTTCTTGC ACTCGGCATAGGCGAGTGCTAAGAAATAACGTT**

EcoRV

4021 **GGCACTCGATATCTGACACTTGAGGAGGAGAGCAAAATTCATGTGCGCCTGGACAAG**
4081 **TCGAAGGTCATCAACTCGGCCCTGGCCCTGGGCAACGAGGTGGGCATCGAGGGCGTGACC**
4141 **ACCCGCAAGCTGGCCCAGAAGCTGGGCGTCGAGCAGCCGACCCTGTACTGGCAGTGAAG**
4201 **AACAAGCGCGCCCTGCTGGACGCCCTGGCCGTGGAGATCCTGGCCCGCCACCACGACTAC**
4261 **TCGCTGCCGGCCGCGAGGCGAGTCGTGGCAGTCGTTCCTGCGCAACAACGCCATGTCGTTCC**
4321 **CGCCGGGCGCTGCTGCGCTACCGCGACGGCGCGAAGGTGCACCTGGGCACCCGCCGGAT**
4381 **GAGAAGCAGTACGACACCGTGGAGACCCAACCTGCGCTTCATGACCGAGAACGGCTTCTCG**
4441 **CTGCGCGACGGCCTGTACGCCATCTCGGCCGTGTCGCACTTCACCCTGGGCGCGGTGCTG**
4501 **GAGCAGCAGGAGCACACCGCCGCCCTGACCGACCGCCCGGACGCCCGGACGAGAACCTG**
4561 **CCGCCGCTGCTGCGGGAGGCCCTGCAAATCATGGACTCGGACGACGGCGAGCAGGCGTTC**
4621 **CTGCACGGCCTGGAGTCGCTGATCCGCGGCTTCGAGGTGCAACTGACCGCCCTGCTGCAA**
4681 **ATCGTGTGAGGGCCCAGCGAGGAGCCCGGTCCTTTGTGGGGCCGGCTCCTCTGGTTG**

XbaI

4741 **GTA**CTCTAGAGGATCCTGTGCGTTTCGCACGCACAGGCCCGGTGTGAGAAGGGTCTCTGCA
4801 **GAGCGGGAGA**ACTCCCTATCAGTGATAGAGTTTGTCTCCCTATCAGTGATAGATAGGCT
4861 **CTGGGAGTACCCGTGTGTACGACCAGCACGGCATAACATTTTCGACGCCGAGAGATTCC**

EcoRI

4921 **CCGCCGAAATGAGCACGAATTC**TGACACTTGAGGAGGAGAGCAAAATTCATGGGGTTA
4981 **CGTAATCGATATGGGCGCGCGCCCTCGATCC**TGCGCGGCGAGAAGCTGGACAAGTGGGA
5041 **AAAGATCCGCTGCGCCCGGGCGGCAAAAAGCACTACATGCTGAAGCACATCGTGTGGGC**
5101 **CTCGCGCGAGCTGGAACGCTTCGCCCTGAACCCCGGCTGCTGGAAACCTCGGAAGGCTG**
5161 **CAAGCAGATCATGAAGCAGTTGCAGCCGGCCTTGCAGACCGGCACCGAAGAGCTGAAGTC**
5221 **GCTGTACAACACCGTGGCCACCCTGTACTGCGTGCACGAGAAGATCGAAGTGC GCGACAC**
5281 **CAAGGAAGCCCTGGACAAGATCGAAGAAGAACAAGAACAAGTGCCAGCAGAAGACCCAGCA**
5341 **GGCCAAGGCCGCCGACGGCAAGGTGTGCGAGA**ACTACGGATCCCCGATCGTGCAGAACCT
5401 **GCAAGGCCAGATGGTGCACCAGGCCATCTCGCCGCGCACCCCTGAACGCTGGGTGAAGGT**

5461 GATCGAAGAAAAGGCCTTCTCGCCGGAAGTGATCCCGATGTTACCCGCCCTGTTCGGAAGG
5521 CGCGACCCCGCAGGACCTGAACACCATGCTGAACACCGTGGGCGGCCACCAGGCCCAT
5581 GCAGATGCTGAAGGACACCATCAACGAAGAAGCGGCCGAATGGGACCGCCTGCACCCGGT
5641 GCACGCCGGCCCGATCGCCCCGGGCCAGATGCGCGAGCCCCGCGGCAGCGACATCGCCGG
5701 CACCACCTCGACCCTGCAAGAACAGATCGCCTGGATGACCTCGAACCCCCCGATCCCGGT
5761 GGGCGACATCTACAAGCGCTGGATCATCCTGGGCCTGAACAAGATCGTGCGCATGTACTC
5821 GCCGGTGTTCGATCCTGGACATCCGCCAGGGTCCGAAGGAACCGTTCCGCGACTACGTGGA
5881 CCGCTTCTTCAAGACCCCTGCGCGCCGAGCAGGCCACCCAAGAGGTGAAGAACTGGATGAC
5941 CGACACCCTGCTGGTGCAGAACGCCAACCCCGACTGCAAGACCATCCTGCGCGCCCTGGG
6001 TCCCGGCGCGACCCTGGAAGAGATGATGACCCGCTGCCAGGGCGTGGGCGGCCCGGGCCA
6061 CAAGGCCCGCGTGTGAATATTGCCGAAGCCATGTTCGAGACCAACTCGGGCAACATCAT
6121 GATGCAGCGCTCGAACTTCAAGGGTCCGCGCCGCATCGTGAAGTGCTTCAACTGCGGCAA
6181 GGAAGGCCACATCGCCCCGCAACTGCCGCGCCCCGCGCAAGAAAGGCTGCTGGAAGTGTGG
6241 CAAGGAAGGTCATCAGATGAAGGACTGCACCGAGCGCCAGGCCAACTTCCTGGGCAAGAT
6301 CTGGCCGTTCGCACAAGGGCCGCCCGGGCAACTTCCTGCAAAAACCGCCGGAACCGACCGC
6361 CCCGCCGGCCGAGTCGTTCGCTTCGAAGAGACCACCCCGGCCCGAAGCAGGAACCGAT
6421 CGAGCGCGAACCCTGACCTCGCTGAAGAGCCTGTTCGGCTCGGACCCGCTGTTCGAGGA

EcoRV

6481 TATCGGCGCCCCGCTGCGCGGCCCGGCCGCGCCTTCGTGACCATCACCTCAAGCAGAT

HpaI

6541 CCCGAACCCCTGCTGGGCCTGGACCAGGGTTAACTAGCGTACGATCGACTGCCAGGCAT
6601 CAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTGTTTGTTC
6661 GGCCATCATGGCCGCGGTGATCA

D6 pPM4RT sequence: Showing the following fragments highlighted in:

- TetRr1.12 cassette: in pink
- Psmc* promoter: blue,
- HIV-1 *rt* gene: green,
- V3-SV5 epitope: grey and

The deleted fragment underlined

1 GCTAGCAACAAAGCGACGTTGTGTCTCAAATCTCTGATGTTACATTGCACAAGATAAAA
61 ATATATCATCATGAACAATAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTA
121 TGAGCCATATTC AACGGGAAACGCTTGTCTCGAGGCCGCGATTAAATCCAACATGGATG
181 CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCT
241 ATCGCTTGTATGGGAAGCCCCATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCG
301 TTGCCAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTC
361 TTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCCTGCGA
421 TCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATATTG
481 TTGATGCGCTGGCAGTGTTCCCTGCGCCGGTTGCATTTCGATTCTGTTTGTAAATGTCTCT
541 TTAACAGCGATCGCGTATTTTCGTCTCGCTCAGGCGCAATCAGCAATGAATAACGGTTTGG
601 TTGATGCGAGTGATTTTGTATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAA
661 AAATGCATAATCTTTTGCCATTCTCACCAGGATTCAGTCGTCACCTCATGGTGATTTCTCAC
721 TTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCC
781 GAATCGCAGACCGATAACCAGGATCTTGCCATCCTATGGAAGTGCCTCGGTGAGTTTTCTC
841 CTTCAATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAAT
901 TGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAAC

961 ACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAAATCGAACTTT
1021 TGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGCAAA
1081 GCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTC
1141 CCTCACTTTCTGGCTGGATGATGGGGCGATTAGGCCTGGTATGAGTCAGCAACACCTTC
1201 TTCACGAGGCAGACCTCACTAGTTCCACTGAGCGTCAGACCCCGTAGAAAAAGATCAAAGG
1261 ATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAAACAAAAAACACC
1321 GCTACCAGCGGTGGTTTTGTTTGGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAC
1381 TGGCTTCAGCAGAGCGCAGATACCAAATACTGTCTTCTAGTGTAGCCGTAGTTAGGCCA
1441 CCACTTCAAGAACTCTGTAGCACCGCCTACATACTCGCTCTGCTAATCCTGTTACCAGT
1501 GGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACC
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XbaI

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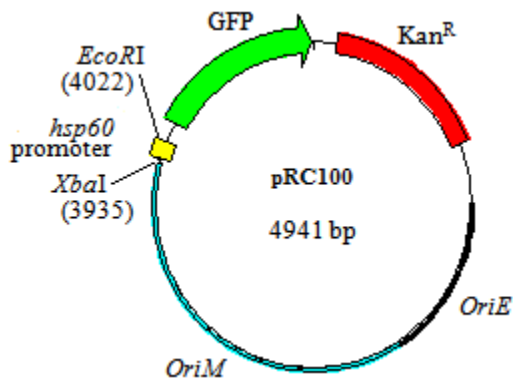
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HpaI

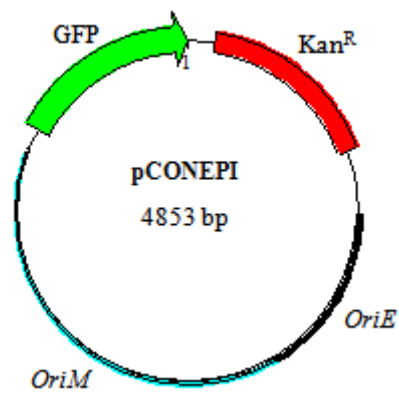
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APPENDIX E: PLASMID MAPS

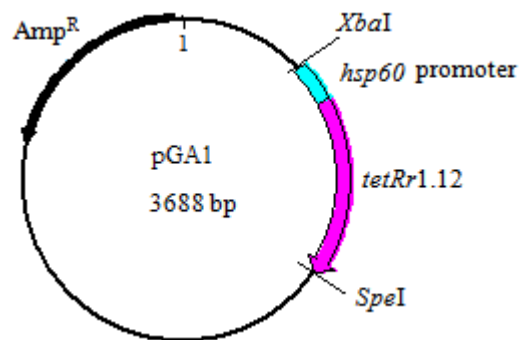
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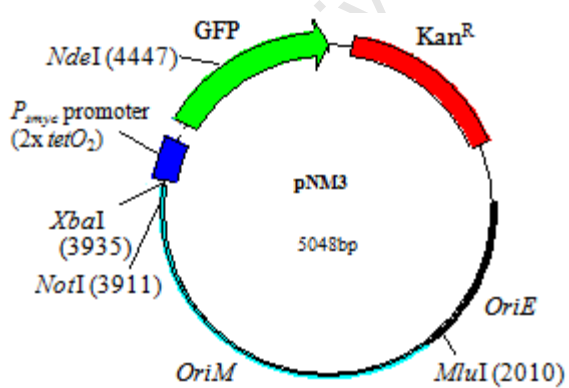
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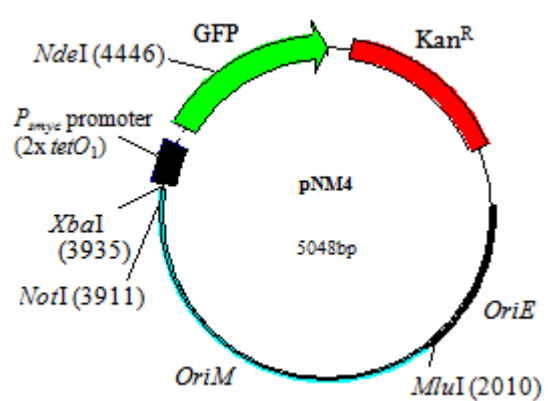
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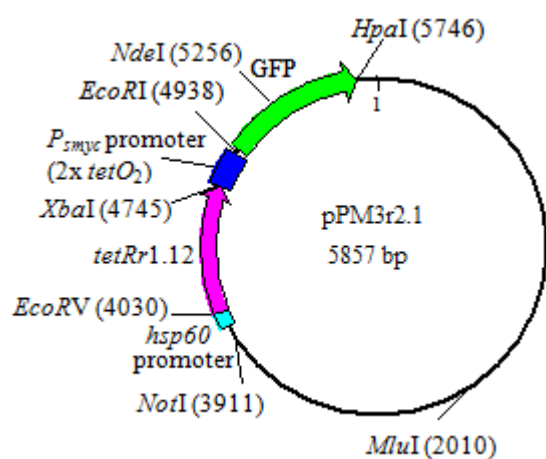
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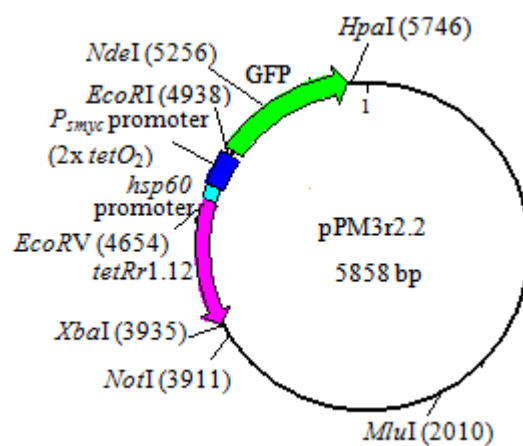
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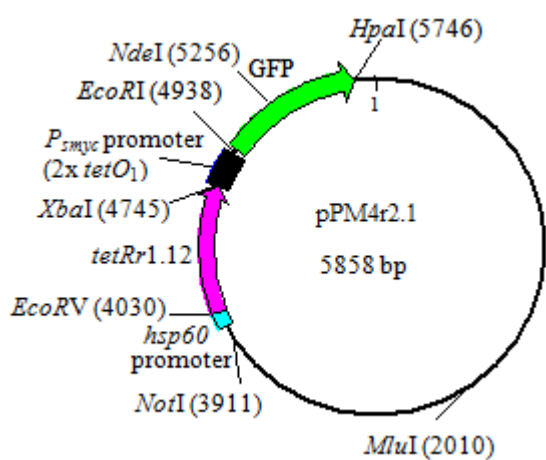
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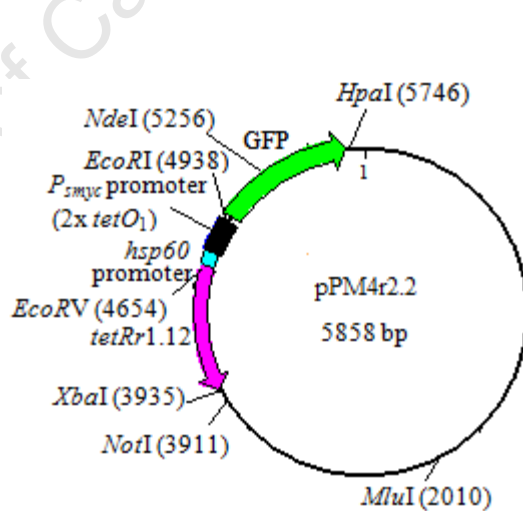
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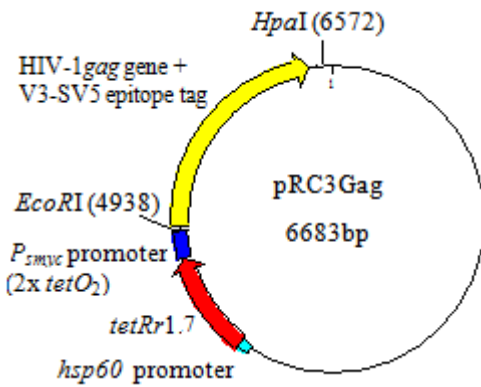
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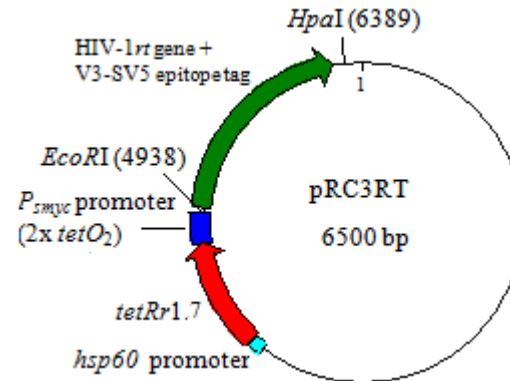
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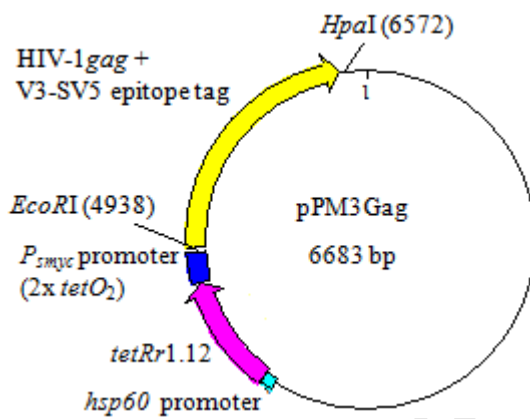
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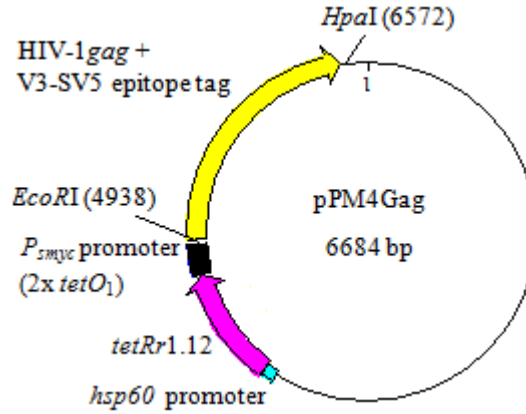
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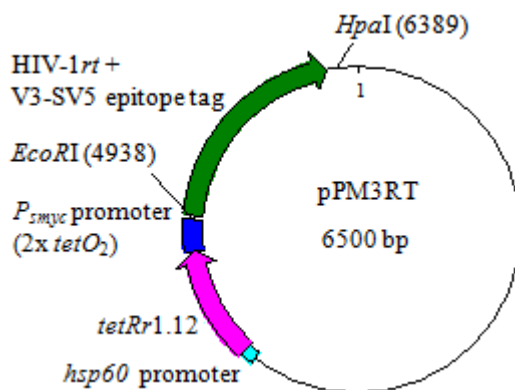
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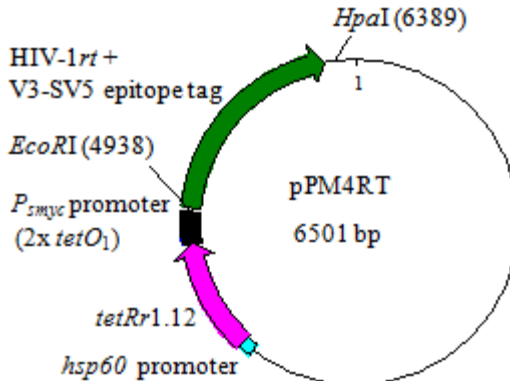
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14.



15.



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