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Contribution of phages to the virulence of pathogenic mycobacteria

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CONTRIBUTION OF PHAGES
TO THE
VIRULENCE OF PATHOGENIC MYCOBACTERIA

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A dissertation submitted to the faculty of Science, University of the Witwatersrand, in the fulfillment of the requirements for the degree of Master of Science.

Johannesburg, 2007

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

(Signature of Candidate)

_____ **Day of** _____ **2007**

ABSTRACT

Bacteriophages have been known to contribute to the bacterial phenotype through lysogenic conversion. Many virulence factors in pathogenic bacteria are phage encoded. However, it is not known whether this is true in mycobacteria. A study by Pedulla *et al,* (2003) looking at the genome sequences of 10 mycobacteriophages suggested that several of the identified genes may have patho-adaptive potential. Perhaps paradoxically, sequenced mycobacterial genomes revealed a paucity of recognizable prophages. To initiate any enquiry into the contribution of prophages to the relevance of mycobacterial disease, we set up some experiments to screen for the presence of prophages in *Mycobacterium bovis* isolates from different outbreaks. We screened 27 isolates for spontaneously induced phages by plaque assay using *M. smegmatis, M. fortuitum, M. scrofulaceum, and M. kansasii* and an isolate (S2) from our lab as indicator strains. However none of these formed reproducible plaques. Only three isolates formed plaques that could not be propagated on any of the indicator strains used. To address if we could enrich for induced prophages, we did some preliminary experiments to optimize prophage induction, using a known lysogen (L5). Co-culturing of a lysogen with sensitive cells was assessed at different concentrations. The result showed that there was no difference in the rate of phage released between the co-cultured and the non-spiked control cells. Since it is possible that we did not have a strain that is sensitive to *M. bovis* phage(s), we checked, using the L5 lysogen, if any free phage could be detected from solid culture, by Epiflourescence Microscopy (EFM). We were able to detect phage particles in a titer of 10^2 as determined by plaque assay with EFM. We therefore screened 16 *M. bovis* isolates for any free phage, using the more sensitive EFM and no inducible phages were detected. Since potential lysogens may be very stable, with minimal induction, we decided to explore a molecular approach to screen for cryptic prophages. Guessmers based on conserved regions in the L5 repressor, shared by other phage genomes were designed .Out of 45 *M .bovis* isolates screened by PCR, nine produced DNA bands of different sizes from each isolate. The sequences from the L5-*M. smegmatis* mc^2155 lysogen positive control were confirmed to be of the gp71 origins (L5

phage repressor).. Sequences from clone DNA from two isolates revealed existence of different *M. bovis* AF2122/97 DNA specific binding proteins such as putative transposases of the IS1553 element (Mb2968) , DNA helicases (Mb0884), transcriptional regulators (Mb1160), and other *M. bovis* proteins such as CTP synthetase (Mb 1725), spermidine synthetase (Mb2632), and a hypothetical protein Mb1618c. Interestingly, a sequence DLLIRVNE which is conserved in L5 and other mycobacteriophage repressor proteins was also conserved in some of the *M. bovis* DNA binding proteins. Hence this protein might play an important role in DNA binding. An in-depth analysis of the whole genome of *M. bovis* is needed in order to conclude if this sequence is of prophage origin.

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LIST OF ABBREVIATIONS

ARC	Agricultural Research Council
Cfu	Colony forming units
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxytyrosine triphosphate
EFM	Epiflourescence Microscopy
KNP	Kruger National Park
L5	Mycobacteriophage L5
MOI	Multiplicity Of Infection
ORF	Open Reading Frame
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase Chain Reaction
Phage	Bacteriophage
Pfu	Plaque forming unit
SA	South Africa
TB	Tuberculosis
TEM	Transmission Electron Microscopy
UV	Ultra Violet
WHO	World Health Organisation

CHAPTER ONE – INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Tuberculosis is still a major global and public health problem, killing more people than any other single infectious agent. *Mycobacterium tuberculosis* a facultative intracellular pathogen is a leading cause of morbidity and mortality in AIDS patients. The situation is worsened by the infection of these immuno-compromised people with *Mycobacterium avium* complex strains. Co-infection of HIV with TB bacilli often leads to progression of one of the diseases. In 2006 the World Health Organisation (WHO) reported that the global TB incidence to be growing by 1% a year and about 2 billion people (third of the world population) are infected with it. They reported that one out of ten infected people will develop active TB. In addition, 20-50 cases of pulmonary TB, using smear microscopy can be missed (Zink and Nerlich, 2004) especially in people co-infected with TB and HIV. Misdiagnosis of TB can also happen because of normal radiographs in HIV positive patients. Bovine tuberculosis, caused by *Mycobacterium bovis* remains an important animal and zoonotic disease causing a significant loss in agriculture world wide, and a public health hazard (Cosivi *et al.,* 1998; Morris *et al.,* 1994). In order to eradicate the disease, it is very important to understand its pathology and what parts of its genetic instructions make the bacillus so virulent.

Tuberculosis studies have lagged behind those of other infectious organisms, because of the slow growth rate of the bacteria. However, recent advances in molecular biology, in particular the elucidation of the genome sequences of both *Mycobacterium bovis* (Garnier *et al.,* 2003) and *Mycobacterium tuberculosis* H37Rv strain (Cole *et al.,* 1998) as well as the CDC155 strain (Fleischmann *et al.,* 2002) has contributed to a better understanding of the pathology and some of the virulence attributes of these organisms.

1.2 LITERATURE REVIEW

The genus *Mycobacterium*

Mycobacteria are a group of Gram-positive bacteria, displaying diverse phenotypes. The genus *Mycobacterium* contains more than 71 recognized or proposed species (Shinnick and Good, 1994). The early classification of mycobacteria was based on growth rate, pigmentation and clinical significance (Runyonn, 1959). A fundamental taxonomic division was tied to growth rate. These can be divided into two main groups based on their growth rate: the fast growers and the slow growers (Shinnick and Good, 1994). Rapid or fast growers include environmental mycobacteria such as *M. smegmatis* (Shinnick and Good, 1994). *Mycobacterium fortuitum* and *M. chelonei,* which can be opportunistic pathogens, are also classified as rapid growers (Ward, 1975). Most species in this group are free-living environmental organisms that rarely cause or never cause diseases.

Among the slow growing mycobacteria are the ubiquitous and facultative pathogenic *Mycobacterium intracellulare* and *Mycobacterium avium, M. avium* subsp *paratuberculosis,* and *M. sylvaticum* which belong to the *M. avium* complex (MAC) (Cangelosi *et al.,* 2001; Mackintosh *et al.,* 2004). MAC bacilli are a group of related environmental mycobacteria. They are common in surface waters and soils and are often isolated from water taps (Kansal *et al.,* 1998; Cangelosi *et al.,* 2001). The host to host transmission mechanism is not known, but exposure to MAC through infected water is common (Kansal *et al.,* 1998; Cangelosi *et al.,* 2001). *Mycobacterium avium* and *M. intracellulare* are commonly associated with human infections by MAC (Wolinski, 1979; Inderlied *et al.,* 1993). *Mycobacterium paratuberculosis* causes Johne's disease in livestock and has been implicated in Chron's disease in humans (Herman-Taylor, 2001).

Several pathogens in the *M. tuberculosis* complex which include *M. tuberculosis, M. bovis, M. microti, M. canettii, M. pinnipedii, M. africanum, M. caprae* also belong to the

slow growing subclass (Brosch *et al.,* 2002; Smith, 2006). This number is likely to increase as new genetic differences between strains of the existing members are identified. The *M. tuberculosis* complex is characterized by a 85%-99.9% similarity at nucleotide level, and identical 16S rRNA gene sequences as determined by DNA sequencing and related methods such as hybridization (Boddinghaus *et al.,* 1990; Sreevatsan *et al.,* 1997; Van Soolingen *et al.,* 1997). The existence of chromosomal deletions together with single nucleotide polymorphism (SNP) and direct repeat content (spoligotype) patterns allows discrimination between these bacteria (Kammerbeek *et al.,* 1997; Brosch *et al.,* 2002; Mostowy *et al.,* 2002; Smith *et al.,* 2003). Synonymous single-nucleotide polymorphism data suggests that *M. bovis,* the primary cause of bovine tuberculosis, evolved at the same time as *M. tuberculosis,* the primary cause of human tuberculosis (Sreevatsan *et al.,* 1997). The study of deletions and insertions in the genomes of *M. tuberculosis* complex provide a strong evidence for the independent evolution of both *M. bovis* and *M. tuberculosis* from another precursor, possibly *M. canettii* (Brosch *et al.,* 2002). More recently sequence analysis of six housekeeping genes (*katG, gyrB, gyrA, rpoB, hsp65, sodA*) revealed that human isolates of *M. canettii* from East Africa represent an extant progenitor of an ancestral species named *M. prototuberculosis,* from which *M. tuberculosis* complex evolved (Gutierrez *et al.,* 2005., Smith, 2006).

The recent elucidation of the genome sequence *M. bovis,* the causative agent of tuberculosis in a range of animals with zoonotic potential (Morris and Pfeifer, 1994; Cosivi *et al.,* 1998) compared with that of *M. tuberculosis,* the primary cause of human tuberculosis, show that these bacilli are genetically similar at the nucleotide level (O' Reilly *et al.,* 1995, Cole *et al.,* 1998; Garnier *et. al.,* 2003). *Mycobacterium bovis* is also the progenitor of the only available human TB vaccine, *M. bovis* bacillus Calmette-Gùrerin (BCG), a strain that was attenuated by serial passaging of *M. bovis* on potato slices soaked in ox-bile and glycerol over 13 years .The precise mutations that led to the attenuation of this strain are still unknown, though the key deletion of the region of difference (RD1) appears to have played a role (Behr *et al.,* 1999; Pym *et al.,* 2002). This hypothesis came about when Mahairas *et al*. (1996) compared the genomic sequences of

M. bovis and *M. bovis* BCG using subtractive hybridization, and found three regions of difference (RD1 , RD2 and RD3) that were absent in BCG, but present in *M. bovis* genomes. Later Behr *et al.* (1999) and Gordon *et al.* (2001) identified 16 large deletions including RD1-RD3 that were present in *M. tuberculosis* but missing in *M. bovis* BCG. Eleven of these 16 deletions were unique to *M. bovis* whereas the remaining 5 were unique to *M. bovis* BCG. One of these (RD1) was absent to all the *M. bovis* BCG strains currently used as vaccine for human tuberculosis.

Epidemiology and Pathology of Bovine Tuberculosis

The main hosts of *M. bovis* are both animals of agricultural importance and wild mammals. Many wild life animals around the world have been infected by this pathogen (Barlow, 1993; White and Harris, 1995; Schmit *et al.,* 1997). Presently there are no available methods for treating wild life population infected with the bacillus, and there is no convincing evidence that these animals are able to resolve the pathogen naturally (O'Reilly and Darbon, 1995; Michel *et al.,* 2005). Once bovine tuberculosis has established itself in a native free-ranging maintenance host, eradication of the disease becomes highly unlikely (Michel *et al.,* 2005).

The choice of suitable control measures for this disease depends on the primary objectives of that particular ecosystem. However various implications have to be considered when choosing a control strategy. These include the preservation of protected species, the minimization of risk of transmission to domestic animals, and the potentially devastating impact on population dynamics (Michel *et al.,* 2005). In New Zealand for instance, the control of bovine tuberculosis is made problematic by the presence of an important wild life reservoir of the disease, notably brushtail possums (Coleman and Cooke, 2001). The absence of effective vaccine strategies for bovine TB, particularly in developing countries has resulted in a test and slaughter policy as the backbone of national elimination programs for this disease (Cosivi *et al.,* 1998). Currently the gold standard for control of bovine tuberculosis internationally, relies on its diagnosis by the

intra-dermal tuberculin test. The main disadvantage of this test in Africa is that it requires trained technicians for correct interpretation (Kleeberg, 1960; Cossivi *et al.,* 1998).

Human infections by this organism in countries where there are no control measures occur when infected, unpasteurized milk or meat is ingested, or when there is close contact with animals that have the disease (Collins and Grange, 1987; Cosivi *et al.,* 1998). The incidence of this kind of tuberculosis in humans is much lower than the disease caused by the human tubercle bacillus (Collins, 2000). Extra–pulmonary tuberculosis is almost always due to drinking infected cow's milk (Kazwala, 1999; Coetzer *et al.,* 2005). The AIDS epidemic has increased the risk of transmission of *M. bovis* to humans (Grange *et al.,* 1994). Human Immune Virus (HIV) infection results in humans becoming more susceptible to all forms of tuberculosis. This not only poses a risk of infection of other humans, but results in livestock being exposed to higher levels of TB. Transmission of this bacillus from humans to animals may also occur. Humans with open tuberculosis caused by *M. bovis* can transmit it to cattle by the aerogenous route, spitting, coughing or urinating (Coetzer *et al.,* 2005).

The first reference of bovine tuberculosis in cattle in South Africa was made by Hutcheon in 1880 (Michel *et al.,* 2005). A potential link between tuberculosis in livestock and game was first suggested, when bovine tuberculosis was reported in kudu and small ungulates in the Eastern Cape Province of South Africa (Paine and Martinaglia, 1929). Subsequently the increasing economic importance of tuberculosis as a disease of cattle led to the implementation of a national bovine tuberculosis control and eradication scheme in South Africa (Paine and Martinaglia, 1929). Bovine tuberculosis was apparently first introduced into African buffalo (*Syncerus caffer*) in the southern regions of Kruger National Park (KNP, South Africa) in the 1960's or 1980's from domestic cattle (Bengis *et al.,* 1996).

Animal to animal (or animal to human) infections by *M. bovis* most often occur when droplets carrying the bacterial cells are inhaled (Collins, 2000). This notion came about because, when the disease develops, the associated granulomatous pathological changes

are seen mainly in the upper and lower respiratory tract (Neill *et al.,* 1994) and because of this pattern it is considered that infection most often follows aerosol exposure to *M. bovis*. The congenital route is also important, and calves may be born with bovine tuberculosis (Cousins *et al.,* 2004), or oral infection can occur in nursing calves (Neill *et al.,* 1994). Infective bacilli gain access to macrophages via inhaled droplets containing the organism. Bacteria not initially killed multiply in the phagosome of macrophages (Adwell *et al.,* 1997). The surviving bacterial particles from bactericidal attack by activated macrophages will infect freshly recruited monocytes or macrophages. When the pathogen reaches sufficient numbers, the cell dies and the released organisms are ingested by other macrophages. The infected macrophages produce cytokines and chemokines that attract other phagocytic cells including monocytes, other alveolar macrophages and neutrophils which eventually form nodular granulomatous structure called tubercle lesions (Dannenberg and Rook, 1994; Fenton and Vermeulen, 1996; van Crevel *et al.,* 2002). The organism is eventually disseminated to the lymph nodes and blood stream. It is deposited in the liver, spleen, kidney, bone, brain, meninges, and other parts of the lung. Infected animals can remain for years without any signs of infection, even in advanced stages, until they are sent for slaughter (Collins *et al.,* 2000).

An important characteristic of pathogenic bacteria is the ability to adapt to a wide range of changing environmental conditions during the infection cycle. *Mycobacterium bovis* demonstrate a remarkable ability to survive in diverse environments. It can survive for years in stationary phase cultures *in vitro*. Outside its hosts this tubercle bacillus remains viable in infective droplet nuclei (Mehrota and Bishai, 2001). *Mycobacterium bovis* can survive for up to two years in soil or manure and possibly even longer in sputum (Kelly *et al.,* 1978; Tanner and Michel, 1999). The pathology and course of infection of *M. bovis* and *M. tuberculosis* is predicted to be very similar (Hart and Sutherland, 1977). In chronic infections, *M. tuberculosis* can remain in a latent state for years, undetected by the host's immune system but ready to switch to an active state once the opportunity arises. This is often dependent on a compromised immune system (Mehrota and Bishai, 2001).

Virulence factors in *M. bovis*

Virulence factors in pathogenic mycobacteria are those attributes that enable the bacterium to infect, survive and multiply in the host's macrophages, resulting in disease symptoms (Collins *et al.,* 2001). The key to the mycobacteria's virulence lies at least in part with their ability to establish residence and proliferate inside the host's macrophages despite the antimicrobial properties of these cells (Dannenberg, 1993; Ernst, 1998). Even though the host mounts a complex immune response, involving both innate and adaptive components that often sequesters the pathogen in granulomas, pathogenic mycobacteria are adept at establishing long-term infections that can manifest as acute or chronic disease or sometimes be clinically asymptomatic with the potential of manifesting at a later stage. An understanding of these factors may lead to strategies for the control of the disease including development of effective vaccines and drugs.

There is no simple answer to 'what makes *M. bovis* virulent? This bacillus does not posses the classic bacterial attributes associated with pathogenecity, like toxins and adherence proteins (Bigi *et al.,* 2005). To define *M. bovis* virulence we need to find factors that are important for the progression of animal TB. A number of structural and physiological properties of this organism are being recognized as contributing to their pathology and virulence (Collins *et al.,* 2001). A review by Cole and Smith (1994) pointed out that there are ongoing studies of these factors, although the studies are still in their infancy possibly because of the pathogen's slow growth rate. The most studied virulence-associated attribute of *M. bovis* is its ability to persist in the host and some of these factors have been reviewed by Smith (2003) and Cosma *et al.,* (2003). They include the bacterial cell envelope function; secretion; its mode of entrance into the host's tissue; cellular metabolism and transcriptional regulation (Smith, 2003; Cosma *et al.,* 2003).

Furthermore, despite the delay in understanding of mycobacterial pathogenicity and virulence, molecular analysis of this tubercle bacillus has undergone a major advancement in the past few years, which has enhanced our knowledge of this bacterium. The elucidation of the genomes of both *M. bovis* (Garnier *et al.,* 2003*)* and *M.*

tuberculosis (Cole *et al.,* 1998; Fleischmann *et al.,* 2002) has allowed the contribution of individual genes to the virulence of these organisms to be determined using comparative genomics. The region RD1 has been identified as a putative virulence factor in both *M. tuberculosis* and *M. bovis* (Behr *et al.,* 1999; Pym *et al.,* 2002). Of the nine genes in RD1, Rv3875 of *M. tuberculosis* encoding Esat6 has been implicated in virulence, since a mutant with a disruption in this gene showed attenuated growth in a guinea pig model (Wards, *et al.,* 2000). Further studies on RD1 might uncover more virulence determinants in this region.

To find genes of selective importance *in vivo*, two approaches have been used to identify genes that are important for virulence: expression screens and mutant screens (Cosma *et al.,* 2003). Expression screens yield important information about the environment. Genes expressed solely in the context of the host can provide information about conditions the bacteria face in the presence of the host's immunity. Genes that are expressed *in vitro* and repressed *in vivo* highlight activities that if expressed, subject the organisms to eradication by the host (Cosma *et al.,* 2003). Therefore there have been parallel efforts to directly identify virulence genes by signature tagged mutagenesis (Tricass and Gicquel, 2000; Glickman *et al.,* 2001; Ainsa *et al.,* 2001). Various strategies have been developed to make mutations in mycobacteria. As it was mentioned, the complete sequence of the genome of *M. bovis* has allowed new genetic approaches into the studies of pathogenicity and physiology of the bacillus. However important work has been done before the elucidation of the genome sequence. These pre-genomic approaches largely dealt with developing methods for creating mutations in specific genes. The choice of which gene to use and ultimately inactivate in order to study virulence, was frequently based on the existence of naturally occurring mutations in normally virulent strains that affected pathogenicity (Collins *et al.,*1995; Wilson *et al.,*1995), or predicted virulence determinants based on clues from other pathogenic bacteria (Smith *et al.,* 1998). Current genetic techniques for inactivating mycobacteria genes have been successful. Gene disruption methods in mycobacteria can be divided into global and directed, but generally require a selectable phenotype, usually resistance to antibiotic. Directed gene disruption entails insertion of an antibiotic resistant cassette in the middle of the gene of interest,

followed by transformation of this DNA to mycobacteria as a linear or circular molecule. The desired result is the allelic replacement of the chromosomal gene by the mutated one (Smith, 2003). In *M. tuberculosis* gene disruption has been made by insertion of long linear DNA (up to 40kb) (Balasubramanian *et al.,* 1996), and shorter ones in the range of 4 kb (Aldovini *et al.,* 1993; Reyrat *et al.,* 1995). Global gene inactivation involves the insertion of a foreign DNA, usually transposable elements in many sites in the mycobacteria genome, ideally in a completely random manner (Smith, 2003).

At first, population genetic studies indicated that; unlike other bacteria, genetic exchange in mycobacteria seems rare. In *M. tuberculosis* and *M. bovis* the unusual structure of RecA, which encodes a key protein responsible for homologous recombination, DNA repair and the regulation of the SOS response (Walker, 1984; Durbach *et al*., 1997), was thought to be responsible for the low frequencies of homologous recombination (McFadden, 1996). This notion came with the limited success of mutagenesis via allelic exchange in these tubercle bacilli (Smith *et al.,* 2003; Supply *et al.,* 2003). The *recA* gene in these mycobacteria is interrupted by an in-frame open reading Frame (ORF) encoding an intein that is removed from a precursor protein by a protein-splicing reaction (Davies *et al.,* 1991; Davis *et al.,* 1992). Subsequent experiments showed that the intein does not affect RecA protein function or the frequency of double cross-over homologous recombination events (Papavinasasundram *et al.,* 1998). Gene knock-outs, using allelic exchange have been successfully achieved in both fast growing and slow growing *mycobacteria.* Recently, Krzywinska *et al.*, (2004) demonstrated evidence for HGT in natural populations of both fast growing *M. smegmatis* and slow growing pathogenic mycobacteria species including *M. bovis*. Subsequently, gene knockout using allelic exchange in *M. smegmatis, M. bovis* BCG and *M. tuberculosis* has been successfully achieved, and it was demonstrated that the recombination frequencies in these organisms were similar to other model bacteria, suggesting that the homologous recombination machinery in fast and slow growing mycobacteria functions with comparable efficiency (Krzywinska *et al.,* 2004). In another study, homologous recombination was demonstrated in *M. bovis* BCG, when a gene encoding orotidine -5' monophosphate decarboxylase (OMPDCase) was isolated from *M. bovis* BCG and transformed to an *E.*

coli mutant lacking this activity. A linear fragment of mycobacterial DNA containing OMPD-Case gene (*ura*A) was then introduced to BCG cells (Aldovini *et al.,* 1993). Pavelka and Jacobs (1999) demonstrated that the recombination frequencies in *M. smegmatis, M. tuberculosis* and *M. bovis* were in fact similar.

Virulence factors of *M. bovis* and *M. tuberculosis* are likely to be similar since these two organisms share 99, 9% of their genes (O' Reilly *et al.,* 1995; Cole *et al.,* 1998, Collins *et al.,* 2001; Garnier *et. al.,* 2003). However as it was mentioned before, deletions in certain RD regions, like deletion in RD9 and RD11 in the *M. bovis* genome, together with the existence of SNP's allows separation of these bacteria (Sreevatsan *et al.,* 1997; Brosch *et al.,* 2002; Parsons *et al.,* 2002). Thus far mutant analysis of mycobacteria has revealed at least four broad categories as described by Cosma *et al.* (2003). These include mutants that are replication-compromised at the onset of infection (e.g. *erp, phoP,* some PG/PGRS loci); others are compromised only later in infection (e.g. *pcaA, and icl*); mutants defective for dissemination (eg *hbhA*); and mutants that are capable of normal replication that confer altered disease pathology (*whiB*3, *sigH*, and *sigC*) (Cosma *et al.,* 2003). Others group them according to their known or predicted functions, like cell secretion and envelope function; enzymes involved in general cellular metabolism; and transcriptional regulation (Smith, 2003).

Recently a new gene *p36,* which is an orthologous gene to *erp* in *M. tuberculosis,* has been identified by Bigi *et al.,* (2005) as a virulence factor in *M. bovis. Erp/P36,* which is specific to pathogenic mycobacteria, is a surface located protein. Its function is not known but has been shown to be a crucial virulence factor since the disruption of its gene impaired multiplication of both virulent *M. tuberculosis* and *M. bovis* BCG in cultured macrophages and immuno-competent mice. This was an indication that the *p36* gene is important for *in vivo* growth in *M. bovis* (Bigi *et al.,* 2005). In Table 1, a few of these virulence determinants are shown and are grouped according to their known or predicted functions.

Table 1: Molecular determinants of virulence in *M. bovis* (Adapted from a review by Smith, 2003)

Virulence Factor	Function	References
CELL SECRETION AND ENVELOPE FUNCTION: Cell walls of pathogenic bacteria are known to show variation in protein sequences and macromolecular composition. Lipids have long been suspected to play a role in mycobacterial pathogenesis. (In this category are genes encoding proteins that are expected to be exposed to the environment , either in culture media or in the macrophages. Among these are proteins and enzymes that play a role in the synthesis of various cell surface molecule (Smith, 2003)		
i) Culture filtrate proteins		
Esat6/CFT-10,(Rv3874,Rv3875).	These are both members of the Esat6 family, immuno-dominant antigens recognized by the sera in majority of TB patients. It is not known which of the two genes Rv3875/Rv3874 located in RD1 are responsible for virulence in *M. bovis* in an animal model used for attenuation	Wards *et al.,* 2000
ii) Cell Surface components		
P36(*p36*)	P36 is a member of family of secreted proteins. Its function is unknown. *P36* gene encodes a secreted 36 kDa protein with a central domain containing several amino acids PGLTS repeats. The authors have shown that disruption of *p36* gene impairs the growth of *M. bovis* in vivo.	Bigi *et al.,* 2005
TRANSCRIPTIONAL REGULATORS: Since Transcriptional regulators control the transcription of many genes, a directed mutational strategy to inactivate regulatory genes would be expected to find some that are important for *M. bovis* virulence as has been demonstrated in other pathogens such as *S. enterica,* serova Typhimurium virulence factors such as sigma factor RpoS and the response regulator PhoP.		
hspR (*hspR*)	*hspR* is a repressor of key heat shock genes like *hsp70.*Physiological experiments showed that *hspR* mutants in both *M. tuberculosis* and *M. bovis* BCG constructed by a two step plasmid technique are depressed for *hsp70* at 37ºC, unlike the wild type strains	McKinney *et al.,* 2000
SigA/RpoV	SigA is a transcriptional regulator in mycobacteria believed to be necessary for the most mycobacterial transcriptional house keeping. Collins *et al* 1995 identified a missense in the SigA. Sig A of *M.bovis* had no effect on growth in vitro, but resulted in attenuation. It was speculated that the attenuation was due to failure of SigA to promote virulence gene expression.	Collins *et al.,* 1995
whiB3	Transcription factor. *whiB3* mutants were created in a virulent *M. bovis* strain. The mutant grew well in vitro, but had notable defects in vivo. The mutant was unable to grow in a mouse model, with a bacterial load 105 lower than the wild type	Steyn *et al.,* 2002
ENZYMES INVOLVED IN CELLULAR METABOLISM: Since many pathogens become starved for essential nutrients and co-factors, like carbon sources, amino acids, purines, pyrimidines, and divalent metals during infection, TB researchers have created mutations in genes encoding enzymes in the biosynthetic/degradative pathways, and acquisition systems for some of these factors		
Nitrate reductase (*narG)*	Plays a role in anaerobic respiration in prokaryotes. A narG mutant of *M. bovis* BCG used to infect mice showed a significant virulence phenotype, whereas its growth under aerobic or anaerobic conditions was unaffected	Weber *et al..,* 2000; Fritz *et al.,* 2002;
GENOMIC COMPARISON STUDIES. The availability of the genomes of both *M. tuberculosis* and *M. bovis* has allowed researchers to investigate potential virulence factors by genomic comparison.		
RD1	RD1 has been implicated in the attenuation of *M. bovis* BCG. Further studies on RDI might lead to the elucidation of more virulence factors.	Pym *et al.,* 2002

Prophages and their Role in Bacterial Pathogenicity: Phage–Mediated Horizontal Gene Transfer.

Bacteriophages are viruses that infect bacteria. They may take up two life cycles upon entering the host: virulent phages only undergo a lytic life cycle, resulting in the lysis of the host and release of progeny phages; whereas temperate phages can undergo both lytic and lysogenic life cycles. Upon entering the host, temperate phages may incorporate their DNA into the host's genome, and form part of the genome and they are termed prophages. Most of the prophage genes can remain dormant in the cell, until they are induced to undergo lytic cycle. However, not all the prophage genes remain dormant. Many of these phages express genes that have subtle effects on the phenotype of the host bacterium (Cianciotto, 1989). Different bacteriophages bring about conversions to their hosts, including increased virulence and pathogenicity (Waldor and Makalanos, 1996). Phage genomes may themselves contain genes valuable to the host (Leitet *et al.,* 2006). In many toxin producing bacteria, pathogenicity and virulence have been shown to be bacteriophage-mediated (Rajadhyaka and Rao, 1965.,Wagner and Waldor, 1996; and Waldor and Makalanos, 1996), which emphasizes their consideration of their role in host pathogenesis and the dissemination of toxin genes amongst different bacterial strains (Wagner and Waldor, 2002). *Vibrio cholera,* Shiga–toxin producing *E. coli, Corynebacterium diphtheria,* and *Chlostridium botulinum,* all depend on specific prophage encoded toxin for causing specific disease (Kar *et al.,* 1995; Brüssow *et al.,* 2004). Furthermore, Shiga toxin encoding phages of *E. coli* have shown that the bacteriophage's life cycle can exert control over virulence factor production by bacterial pathogens. Shiga toxins (Stx1 and Stx2), are the principal virulence factors in enterrohemmorhagic *E. coli.* Stx1 is reported to be located within prophage related to lambda, which contain transcriptional units for various functions such as replication, morphogenesis, and lysis that are coordinately expressed during specific intervals following prophage induction due to regulatory influence of phage promoters, phage repressor, transcriptional terminators and antiterminators (Wagner and Waldor, 2002).

Toxin genes are only a subset of the diverse virulence factors encoded by bacteriophages. Phages can also contribute directly to bacterial virulence (Mavris *et al.,* 1997; Guan *et al.,* 1999). In addition to conferring non-structural components as virulence factors, structural components of the virion may also confer virulence attributes. In two *Shigella flexneri* bacteriophages (SfV and SfII), O-antigen glycolysation was characterized (Huan *et al.,* 1997a, b; Mavris *et al.,* 1997). A three gene cluster was found (*gtrA, gtrB, and* gtrX) in both these O antigen glucolysation bacteriophages. The *gtrA* gene in phage SfV encodes a highly hydrophobic protein of unknown function and *gtrB* in SfII has been allocated a bactoprenol glucose transferase function. A study by Guan *et al.*, (1999) showing molecular characterization of phage SfX of *Shigella flexneri* also demonstrated that the first gene (*gtrA)* of a three gene cluster to be most likely to encode a protein involved in the translocation of lipid linked glucose across the cytoplasmic membrane, leading to a modification on the bacterial cell surface. These authors also showed that the second gene (*gtrB*) encodes a bactoprenol glucose transferase. As protective host immune response to *S. flexneri* infection is directed against the O-antigen. This resulting serotype conversion represents an important virulence factor for the bacteria (Guan *et al.,* 1999).

Some phages encode regulatory factors that increase expression of the virulence genes encoded by the phage, while others encode enzymes that alter bacterial components related to pathogenicity (Wagner and Waldor, 2002). A study by Spanier *et al.,* (1980) showed bacteriophages to be involved in conversion of some streptococci strains to a phagocytosis-resistant phenotype. The M protein is a streptococcal cell surface antigen responsible for phagocytosis by human polymorphonuclear leuckocytes. In this study two genes involved in the synthesis of anti-phagocytic M protein were found, one namely *mprA* contributed by a prophage, and the second *mprS* contributed by the bacterium. The genetic mechanism by which the wild type cells were converted to phagocytocis–resistant phenotype were speculated to involve site specific inversion, deletion and insertion of a controlling DNA segment in the prophage (Spanier *et al.,* 1980).

As mobile elements, bacteriophages can also serve as vectors for transferring genes and therefore potential virulence factors between bacteria. Comparative analysis of the whole genome sequences of most bacteria suggests that many genes have been transferred between prokaryotic species through horizontal gene transfer (HGT) (Ochman *et al.,* 2000). Phage mediated HGT occur via transduction. Generalized transduction is observed with many bacteriophages. It was first demonstrated in *Salmonella typhimurium* with phage P22 by Zinder and Lederberg (1952) and subsequently in *E. coli* with phages P1 (Lennox, 1955) and T1 (Drexler, 1970). Wilson (1979) later described a mutant of T4 that displayed the property of generalized transduction. In the process of generalized transduction, after the empty heads are assembled, the phage DNA must be packaged, but instead there is aberrant packaging of host DNA fragments at a finite frequency. This results in delivery proficient phage particles that will deliver the bacteria DNA fragments into another host. In pathogenic bacteria, HGT is thought to facilitate maintenance and enhancement of virulence as well as the spread of antibiotic resistance (Brüssow *et al.,* 2004).

Phage encoded genes are not always transmissible due to the fact that integrated prophages frequently become defective which may lead to them becoming cryptic since the gene content will decay in the absence of selection. However some of the residual gene content may be retained if it confers a selective advantage to its host. For instance the non-transmissible *stx* genes in *Shigella dysenteriae* are adjacent to lambdoid phage-like sequences interrupted by numerous insertions sequences, suggesting that the toxin genes lie in a prophage that has been rendered defective by these insertion sequences (McDenough *et al.,* 1999). Defective prophages have been identified by analysis of sequenced bacterial genomes. Comparative genomics has provided a tool for prophage identification in sequenced bacterial genomes. Many sequences of bacterial pathogens deposited in the public database contain prophage DNA integrated in the bacterial chromosome. Other bacteria contain multiple prophages that constitute a large part of their genomes (Canchaya *et al.,* 2003). The most extreme cases are *E. coli* 0157:H7 strain Sakai, a human pathogen. It contains 18 prophage genome elements, amounting to 16% of its total genome content (Perna *et al.,* 2001). Other cases are that of Streptococcus

pyogenes genomes of the sequenced M1, M18 and M3 strains with four to six prophages each, which amounts to 12% of the bacterial DNA component (Smoot *et al.,* 2002). Furthermore, prophages have been demonstrated to play a role in inter-strain genetic variation in several related bacterial species. Examples include *Staphylococcus aureus* (Baba *et al.,* 2002) and *S. pyogenes* (Smoot *et al.,* 2002). When genomes from closely related bacteria were compared in a dot plot analysis, prophage sequences frequently accounted for a substantial proportion of the difference in the bacterial strains. Examples are *E. coli* 0157:H7 and K12 strains (Perna *et al.,*2001); *Listeria monocytogenes* and *Listeria innocua; Salmonella enterica* serovars Typhi and Typhimurium (McClelland *et al.,* 2001; Parkhill *et al.,* 2001) Recently Srividhya *et al.* (2006) has used a Protein Similarity Approach (PSA) to identify cryptic prophages. In this approach, using information available from the integrated prophages of sequenced bacterial genomes, they identified prophage e14 (a coliphage) homologs by similarity searches at protein level. They took twenty three e14 proteins as query and used bacterial proteomes as target (Srividhya *et al.,* 2006). The e14 element is a very well characterized prophage element, containing all the highly conserved prophage genes like the integrases, excisionase, phage portal, cro type regulator, repressor and terminase genes elements (Mehta *et al.,* 2004). Using the PSA, Srividhya *et al.,* (2006) identified probable prophages in both pathogenic and non-pathogenic bacterial genomes. Some of these prophages were reported in the literature and there were some with no literature reports. The unreported prophages in the literature, included among the others, putative prophages of *M. bovis* AF2122/97*,* and a prophage from *S. pyogenes* M18 strain.

The role of prophages is not limited to pathogenic bacteria, but some adaptations of non-pathogenic bacteria to their ecological niche are believed to be prophage-mediated (Brüssow and Hendrix, 2002). For instance, it is becoming clear that gut commensals and pathogenic bacteria have much in common. *Lactobacillus* commensal bacteria are under selective pressure of a T–cell independent mucosal IgA and respond by changing their surface polysaccharide. Sequenced *Lactobacillus* commensals contain multiple prophage genomes that showed lysogenic conversion genes related to those of the prophage of *S. pyogenes.* In addition, the dairy strain *Lactobacillus lactis* IL1403 contains six prophage

genomes (Chopin *et al.,* 2001). One therefore might suspect that these prophages contribute to the evolutionary success of lactic acid bacteria living in strikingly distinct environments (Brüssow and Hendrix, 2002).

HGT in pathogenic mycobacteria

Recombination between different strains of *M. tuberculosis* must be rare since the bacteria rarely come into contact with different populations in the context of an infection (Smith *et al.,* 2003; Supply *et al.,* 2003). Because of its isolated living space, lack of migration between hosts, long generation time, and latent infections, members of the *M. tuberculosis* complex remain paradigms of clonal evolution. Thus if recombination occurs, it happened between identical individuals and hence leaves little detectable traces (Smith *et al.,* 2003). However, the presence of different gene delivery mechanisms and functional homologous recombination machinery in *M. bovis* and other members of mycobacterium species raise the possibilities of naturally occurring transfer and recombination. It has been shown that mycobacterial plasmids can replicate within most *Mycobacterium* species, so they can theoretically be spread horizontally, promoting gene transfer between mycobacteria (Le Dantec *et al.,* 2001; Kirby *et al.,* 2002). Patients simultaneously infected by two different strains of *M. tuberculosis* have been reported (Shafer *et al.,* 1995; Theisen *et al.,* 1995; Yeh *et al.,* 1999; Pavlic *et al.,* 1999; Braden *et al.,* 2001; Das *et al.,* 2004). A chromosomally coded conjugation system has recently been identified in *Mycobacterium smegmatis* (Parsons *et al.,* 1998; Wang *et al.,* 2003) which opened up the possibility of lateral DNA transfer, via conjugation with other mycobacteria. Furthermore several plasmids were found in *M. avium* complex (Meissner and Falkinham, 1986; Hellyer *et al.,* 1991) among them plasmid pVT2, which is thought to be conjugative (Kirby *et al.,* 2002) and raises the possibility of conjugational transfer in *Mycobacterium avium* complex. Evidence of recombination has been shown in *M. tuberculosis* strains isolated from TB patients in East Africa (Gutierrez *et al.,* 2005). In these isolates, incongruence among gene phylogenies as well as mosaic gene sequences, whose individual elements are retrieved in classical *M. tuberculosis* were detected. Therefore, despite its apparent homogeneity, the *M. tuberculosis* genome appears to be a

composite assembly resulting from horizontal gene transfer events predating clonal expansion (Gutierrez *et al.,* 2005).

Possible Role of phages in HGT in Mycobacteria

Mycobacteriophages are extremely diverse in nature and carry highly mosaic genomes (Pedulla, *et al.,* 2003). Most of these viruses have been isolated from environmental samples. While many bacteriophages have been isolated from environmental samples, only 30 have been sequenced and characterized so far (Pedulla *et al.*, 2003; Hatfull *et al*., 2006). Genomic comparison of these phages at nucleotide level reveals considerable overall diversity, with small groups having sequence similarity. The most numerous phage genome clusters contains seven that are more closely related to each other than the others phages. These include: L5, Bxb1, D29, Bethlehem, U2, BxZ2, and Che12. The next numerous phage cluster contain six phages. These include Rosebush, Orion, PG1, Cooper, Qyrzula and Pipefish. Another cluster is formed by phages Che8, PMC and Llij (Hatfull *et al.,* 2006). L5, Bxb1, D29 and TM4 have similar arrangement of structure and assembly genes. The two most closely related genomes are those of L5 and D29, sharing over 75% of their genes at amino acid level. More than 40% of the predicted protein products of Bxb1 are related to those of L5 and D29 (Pedulla *et al.,* 2003). The nucleotide sequence comparison of these 30 sequenced mycobacteriophages is shown in a dotter plot in Fig 1 below.

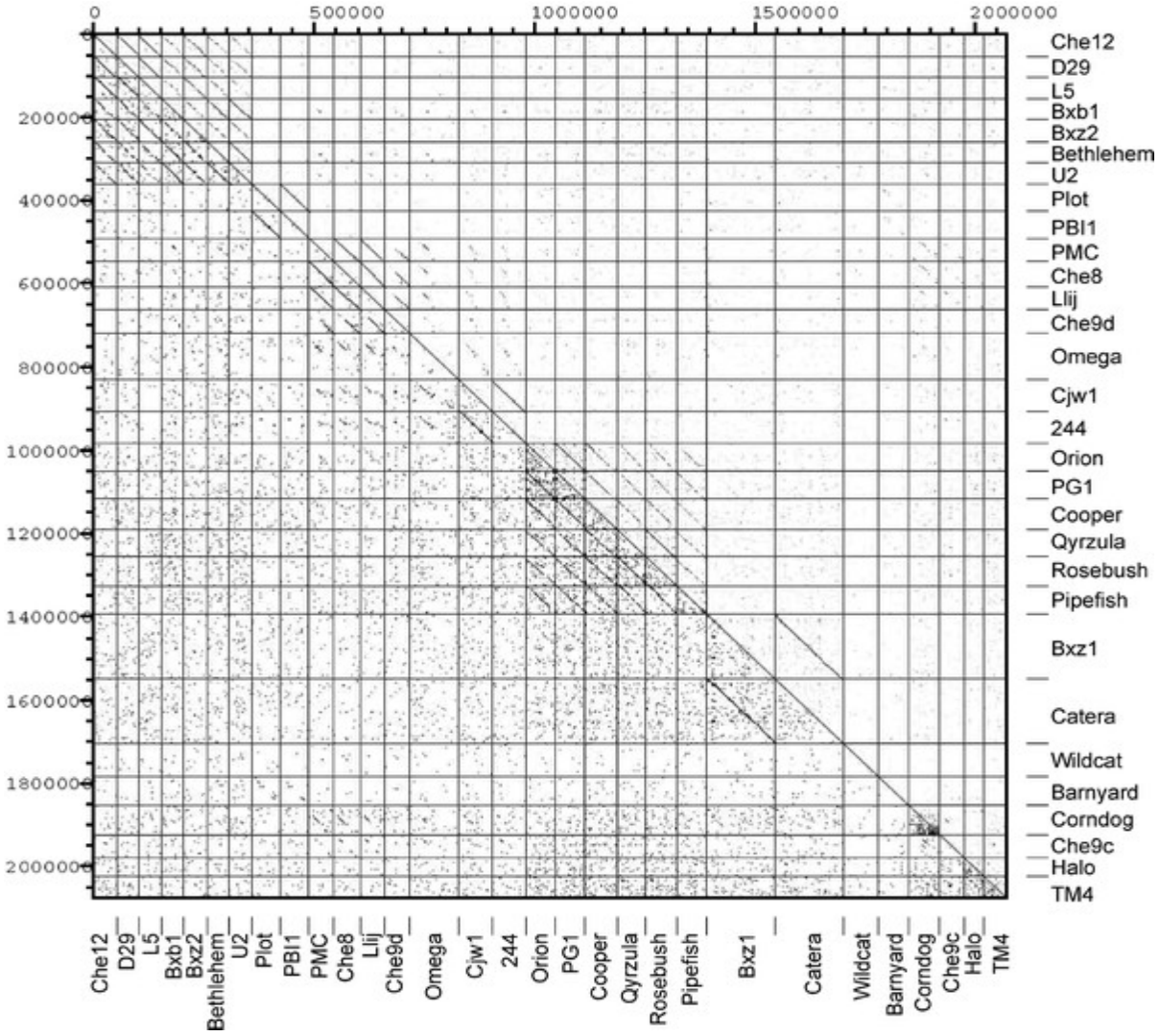

Fig 1: Nucleotide sequence comparison of 30 mycobacteriophages as illustrated in a Dotter plot using a sliding window of 25bp. The lower triangle represents relationships at an elevated level of grey- scale relative to the upper triangle, revealing weaker sequence relationships. DOI 10.1371/journal.pgen. 0020092.g001 (extracted from Hatfull *et al.,* 2006)

Genomic characterization of 14 of these mycobacteriophages revealed within their genomes many unexpected genes that were not previously thought to be phage encoded (Pedulla *et al.,* 2003). Some of these phage genes, for example gene 39 and 69 of phage CjW1 and Omega respectively are reported to encode close homologs of *M. leprae* and *M. tuberculosis* immuno-dominant antigen Lsr2 that is a potent stimulator of both cellular and humoral immune responses. Even though the function of Lsr2 protein is not known it

is likely that these viruses could influence immune response of their host through the introduction of these genes (Pedulla *et al.,* 2003), hinting at a possible role of phages in mycobacterial virulence. This also suggests the possible involvement of these phages in genetic exchange between the prokaryotes, including gene transfer among mycobacteria. In addition, the genome sequences of mycobacteriophages have shown that phage genomes are extensively highly mosaic in nature with regions of obvious sequence similarities interspread with segments that appear to be unrelated, suggesting extensive horizontal genetic exchange among these viruses (Hendrix *et al.,* 1999; Pedulla *et al.,* 2003). At first it was postulated that phages evolve by genetic exchange at special intergenic sites, either through homologous recombination or by site specific mechanism (Susskind and Botstein, 1978). However, the recent elucidation of the genomes of several lambdoid phages has allowed the revelation of a different picture of mosaic formation (Juhala *et al.,* 2000; Hendrix, 2002). In this model, illegitimate recombination is believed to take place quasi-randomly along the recombination genomes, generating an unholy mélange of recombinant types, almost all of which will be defective for phage growth as a consequence of their misplaced recombination. Natural selection eliminates all but the tiny minority of recombinants in which biological function is intact, thus giving rise to a observable population in which the sites of recombination are random (Juhala *et al.,* 2000; Hendrix, 2002). It is not clear as to what degree this picture of horizontal exchange extends to other phage groups, however, the availability of the genomes of 14 mycobacteriophages provides an opportunity for the elucidation of the evolutionary mechanisms that generate mosaicism in these mycobacteriophages (Pedulla *et al.,* 2003). Pedulla *et al.,* (2003) postulated that one explanation is that, genetic modules are re-assorted by homologous recombination at short conserved boundary or (linker) sequences, as suggested initially by Susskind and Botstein (1978). An alternative model is that, illegitimate exchange plays the major role, recombining viral and non-viral DNA molecules in a sequence independent manner that generates mostly genomic trash that is either incorrectly sized for packaging into capsid or lacks required genes. The viable genomes that pass this filter for function and size will retain recombination boundaries that have had minimal impact on gene function, occurring either at or close to gene boundaries. The generated mosaic junctions will be subsequently re-assorted by

homologous recombination between flanking sequences (Pedulla *et al.,* 2003). If the mosaicism of phages is generated by illegitimate recombination, then the acquisition of genes from the host is to be expected (Hatfull *et al.,* 2006).

Even though there are no further reports of prophages capable of forming complete phages that are infective in *M. bovis* and *M. tuberculosis*, the availability of the genome sequences of both *M. tuberculosis* and *M. bovis* has allowed the identification of prophage sequences in these tubercle bacilli. The genome of *M. tuberculosis* H37Rv strain has been reported to contain two prophage like-elements, phiRv1 and phiRv2. Both are relatively small (~10kb) but contain several phage related sequences (Hendrix *et al.,* 1999). PhiRv2 has at least two homologues of mycobacteriophages genes: integrase (Rv2659c, which is related to integrases of phages L5 and D29) and a relative of L5 gene *36* (Rv2657c). It also contains a second recombinase, Rv2647, a homologue of the prohead protease genes of phages HK97 (a coliphage) and *Streptomyces* phage ΦC31; and a homologue of the actinophage RP3 (a temperate phage of *Streptomyces*) gene, in addition to several others (Hendrix *et al.,* 1999). In addition to this the phiRv1 also encodes an active site specific recombinase, where an integrase of the serine recombinase family catalyses recombination and excision, while an adjacent small Open Reading Frame (ORF) controls the directionality of recombination (Canchaya *et al.,* 2003). Presumably the recombination functions associated with phiRv1 are active and may therefore mediate the integration and / or excision of this DNA segment (Hendrix *et al.,* 1999). The phiRv2 prophage element also may be recombinationally active because the attachment junctions (*attL* and *attR*) are present and appear to derive from a phage attachment site (*attP*) that is structurally similar to the L5 *attP* site (Pena *et al.,* 1997). Although the two prophages appear too small and too deficient in terms of virion structural genes to encode a complete virion, they could in fact be "complete" satellite phages in the manner of coliphage P4 which uses the structural genes of another phage to package its genome (Hendrix *et al.,* 1999). Prophages phiRv1 and phiRv2 were identified to be contained in regions RD3 and RD11 respectively in *M. tuberculosis.* In a study to identify and differentiate between *M. tuberculosis* complex isolates by PCR deletion analysis, the region RD3 was found to be present in most *M. bovis* isolates (Parsons *et*

al., 2002). The NCBI and the Sanger Institute databases also reveal the presence of phiRv1 in the *M. bovis* AF2122/97 sequenced strain (www.ncbi.nlm.nih.gov; www.sanger.co.za). However as mentioned before, an avirulent *M. bovis* BCG strain has neither phiRv1 nor does it have phiRv2. This strain is thought to have lost these prophage sequences during its attenuation (Parsons *et al.,* 2002) suggesting that these prophages might play a role in the pathogenicity of both *M. bovis* and *M. tuberculosis.*

1.3 AIMS AND OBJECTIVES

Bacteriophages have been known to contribute to the bacterial phenotype through lysogenic conversion. Many virulence factors in pathogenic bacteria are phage encoded. However, it is not known whether this is true in mycobacteria. A study by Pedulla *et al.,* (2003) looking at the genome sequence of 10 mycobacteriophages isolated at the time of the study, suggested that several of the identified genes may have patho-adaptive potential. Perhaps paradoxically, sequenced mycobacterial genomes revealed a paucity of recognizable prophages. A study by Tageldin *et al.,* (1981), which looked at 7 bovine strains, only isolated two spontaneously inducible phages from two strains that had different host range. To initiate an enquiry into the contribution of prophages to the relevance of mycobacterial disease, we set up some experiments to screen for the presence of inducible and or cryptic prophages in *Mycobacterium bovis* isolates from different outbreaks. Using a well-characterized phage system (mycobacteriophage L5), bacteriophage isolation methods were optimized and tested on the *M. bovis* isolates. Our objectives therefore were:

Objectives

- To detect spontaneously induced prophages using more than one sensitive indicator strains
- To screen for the presence of spontaneously induced prophages using non-selective methods
- Identify prophages using a genomics approach

CHAPTER TWO- SCREENING FOR SPONTANEOUSLY INDUCED PHAGES FROM *M. BOVIS* ISOLATES.

2.1 INTRODUCTION

Spontaneous phage induction is a process that results in the conversion of a prophage into a lytic phage, without the use of chemical or physical inducing agents. It is the characteristic of a temperate phage to either enter a lysogenic or dormant state or undergo lytic infections. Prophages are integrated viral genomes in bacteria and range from fully viable, to cryptic prophages that have undergone mutational decay and do not result in lytic growth. Prophages can often be induced from their host by UV or chemical treatment.

There are essentially two approaches to studying inducible phage populations associated with bacteria. Those approaches that involve direct isolation and therefore require sensitive strains (Tokunga *et al.,* 1971; Tageldin *et al.,* 1981; Kilic *et al.,* 2001). The other approach, involving microscopy, simply identifies the presence of phage, but does not attempt to isolate it. These microscopy approaches more traditionally involved transmission electron microscopy (TEM) (Rieber and Imaeda, 1969; Bergh *et al.,* 1989; Borsheim *et al.,* 1990; Humphrey *et al.,* 1999; Maggi and Breitsch, 2005), but currently, Epiflourescence microscopy is becoming more popular (Suttle *et al.,* 1990; Hara *et al.,* 1991; Proctor and Fuhrman, 1992; Noble and Fuhrman, 1998). In TEM, the bacterial cells are harvested from liquid culture by centrifugation of the cultures (at 4,000×*g*) (Humphrey *et al.,* 1999) followed by suspension of the bacterial pellets in phage buffer and negative staining for TEM to view any phage particles attached to the bacterial cells, or ultracentrifugation of the supernatant (at 40,000×*g*) (Rieber and Imaeda, 1969) followed by negative staining to view free phage particles in the supernatants. TEM reveals the morphology and size of the bacteriophage (Rieber and Imaeda 1969; Humphrey *et al.,* 1999) where as EFM only reveals the presence of the phages in the sample (Noble and Furhman, 1998). In EFM, phage particles are harvested from bacterial cultures by centrifugation followed by filtration of the supernatants through a 0.02µm

pore size Al_2O_3 Anodisc 25 membrane filter and stained with SYBR Green 1 stain (Noble and Fuhrman, 1998).

Phages have been isolated from pathogenic and non-pathogenic strains of mycobacteria. In 1984 Timme *et al.* isolated bacteriophages from *Mycobacterium avium, Mycobacterium intracellulare* and *Mycobacterium scrofulaceum,* after treatment of bacterial cultures with mitomycin C. In addition lysogeny has been demonstrated in *M. fortuitum* after treatment of the bacterium with mitomycin C and UV induction (Jones, 1973; Grange and Bird, 1975). In 1981 Tageldin *et al.* isolated two mycobacteriophages from lysogenic Sudanese bovine strains. Out of seven bovine strains tested for mycobacteriophage inductions, only two produced plaques. It was also shown that they had different host range (Tageldin *et al.,* 1981).

Most mycobacteriophage studies have employed the use of plaque assay procedures which need sensitive indicator strains for direct isolation and propagation of these phages. The already isolated mycobacteriophages may serve as useful controls for optimizing some experimental parameters. In this study a well characterized mycobacteriophage L5 has been used as a control in some experiments as it is easy to work with, since even though it may form stable lysogens with *M. smegmatis*, as a temperate phage, it is spontaneously inducible (Fullner and Hatfull, 1997; Lee *et al.,* 1991; Snapper *et al.,* 1988). L5 lysogens spontaneously generate free phage particles, by a way of prophage excision (Lewis and Hatfull, 2000). Prophage excision involves site-specific recombination between the attachment junction *attL* and *attR*, and requires a phage encoded excisionase protein in addition to integrase and host factor (Landy, 1989; Lewis and Hatfull, 2000). Establishment of lysogeny involves integration of phage genome to the mycobacterial host chromosomal genome through the integrase- mediated site specific recombination event (Lewis and Hatfull, 2000). Maintenance of lysogeny by temperate phage requires functions of repressor proteins (Lewis and Hatfull, 2000).

2.2 AIMS AND OBJECTIVES

Based on the low levels of phages seen in *M. bovis* using plaque assay (Tageldin *et al.,* 1981), we reasoned that the use of a single sensitive strain might be biased, and hence could only select for phages that can infect that strain. In addition only a few studies as far as we know have attempted to look at the use of more than one indicator strain. We hence decided to screen for the presence of phages from *M. bovis* strains from different outbreaks, using a plaque assay on different indicator strains (selective isolation), as well as a non-selective epifluorescence microscopy. Epifluorescence microscopy allows for the detection of spontaneously induced phages, irrespective of their host specificity. In order to develop a control for these experiments, we used the well characterised L5 phage system.

Our objectives therefore were:

- To test some approaches to attempt to increase the levels of phages in culture filtrates using the well described and characterised phage L5.
- To screen for the presence of spontaneously liberated prophages by periodic sampling of cultured *M. bovis* isolates using a standard plaque assay.
- To screen *M. bovis* isolates for spontaneously inducible prophages by non-selective Epiflourescence microscopy.

2.3 METHODS

Bacteria and phage strains used in this chapter are listed in Table 2 below.

Table 2: List of strains used in this study

Bacteria Strains	
Name	**Source**
M. bovis isolates	Dr. Michel, Agricultural Research Council- TB laboratory[1]
Indicator bacteria strains	
M. fortuitum	Dr Michel, Agricultural Research Council-TB laboratory
M. kansasii	Dr Michel, Agricultural Research Council-TB laboratory
M. scrofulaceum	Dr Michel, Agricultural Research Council-TB laboratory
M. smegmatis- L5 lysogen	Developed by Ms. Belinda Spillings, University of the Witwatersrand, (Dr Durbach's Lab)
M. smegmatis mc^2155 (wild type)	Prof Dabbs, Wits University
Mycobacterium ' S2' isolate[2]	Dr Lukusa Kambulu, University of the Witwatersrand (in Dr Durbach's Lab)[1]
Phage strains	
L5 phage	Ms Spillings, University of the Witwatersrand

[11] ***M. bovis*** **isolates-. Their species and origin is indicated in Table 3 and 4 in the results section.**

[2] **'S2'- mycobacteria isolate =98% similar at amino acid level to *M. smegmatis,* characterized by Dr Lukusa Kambulu**

2.3.1 Liquid culturing of mycobacteria indicator strains

Different mycobacteria indicator strains which were maintained as liquid freezer stocks at -70°C in 15% glycerol (v/v) were cultured in Middlebrook 7H9 medium (Difco) (Appendix B) supplemented with 1mM $CaCl_2$, 1mM $MgCl_2$ (to facilitate phage attachment); 0.03% glucose; 0.2% glycerol; 0.05% Tween 80 to reduce clumping (Snapper *et a*l., 1988) and were then incubated at 37ºC and shaken at 120rpm on a platform shaker. Growth was monitored by optical density analysis at 600nm.

2.3.2 Liquid culturing of *M. bovis* isolate strains

Different *M. bovis* isolates from solid cultures in Löwenstein Jensen (LJ) medium (National Health Laboratory Services) medium were cultured in Middlebrook 7H9 medium (Difco) supplemented with 0.03% glucose by dispensing 5ml aliquots of the medium on the surface of the LJ slant with colonies. The medium containing *M. bovis* was then poured into another 5ml of Middlebrook 7H9. The cultures were then incubated at 37°C and were manually shaken gently for an average of 5 times daily.

2.3.3 Propagation of L5 and preparation of large scale L5 stocks (adapted from Sambrook *et al.*, 1989)

Phage plaques on bacterial indicator lawn were picked by immersing a blue pipette tip on the plaque and sucking. The agar plug containing the phage was suspended in 1ml phage buffer (see appendix B) and mixed by gentle pipetting. The suspensions were left at room temperature to allow phage diffusion from the agar. They were centrifuged at 5000rpm in a benchtop centrifuge for 2 minutes followed by filtration through a 0.45 µm non-pyrogenic hydrophilic disposable filter (Ministart). For large scale phage stocks, the phage suspensions were plated on 20 to 25 *M. smegmatis* mc^2155 or *M. fortuitum* indicator plates or overlays (see sections 2.3.5, and 2.3.6) and incubated at 37º C, until plaques were seen. When plaques appeared, 5ml of phage buffer was poured onto the

plates and left overnight at 4ºC with intermittent gentle shaking to allow diffusion of the phage particles into the phage buffer. The phage buffer was then harvested and placed in 50ml polypropylene Falcon tubes. Then 1ml of phage buffer was again added onto the plates and stored for 15 min in a tilted position to allow the fluid to drain into a localised area. The phage buffer from the plates was again removed and combined with the first harvest. The suspension from the lysate stocks was centrifuged at 3500×*g* for five minutes to pellet cell debris. The supernatant was transferred into 50ml Falcon tubes (Lasec). Sodium chloride to a final concentration of 1M and 10% polyethylene glycol (PEG) were added and gently shaken until dissolved. The solution was stirred at 4ºC overnight in a platform shaker. The formed precipitate was centrifuged at 3500×*g* for 5 minutes at 4ºC. The phage pellet was resuspended in about 4 ml phage buffers, and mixed gently by pipetting up and down. The volume of the suspended phage was measured, and 0.5g/ml (m/v) of Cesium Chloride (CsCl), was added on the phage suspension and mixed to dissolve. The suspension was layered on top of a Cesium Chloride step gradient that was prepared by layering solutions of CsCl dissolved in phage buffer to obtain densities of 1.7g/ml, 1.5g/ml and 1.45g/ml on top of each other in a Beckman SW41 or SW28 clear polyethylene tube. The gradient with the phage suspensions were centrifuged in Beckman SW28 or SW41 rotor at 22000rpm at 4ºC for 2 hours. The phage band between 0.45g/ml and 5g/ml CsCl densities , was collected by either puncturing the side of the tube with the phage using a sterile needle or by inserting a sterile plastic syringe on top of the centrifuge tube and sucking the phage band. The collected phage was dialyzed using a dialysis bag (Seal air) that was soaked in phage buffer overnight at 4ºC. The phage stock was screened for purity and viability and titer determined by plating on *M. smegmatis* mc2155 indicator plates (see section 2.3.4). The phage stock maintained at 4ºC until needed.

2.3.4 Preparation of *M. bovis* phage filtrate

Mycobacterium bovis filtrates were prepared by sampling 5ml from each liquid culture periodically (up to 3 months of incubation) in sterile 50ml Falcon tubes (Lasec). They

were then centrifuged at 1088×*g* for 10min on a benchtop centrifuge (Heraeus). The supernatants were filtered using a 0.45µm filter (see section 2.3.3).

2.3.5 Preparation of Indicator Plates for phage detection (Adapted from McNerney *et al.,* 2004)

Indicator plates were prepared by addition of a 10% (v/v) indicator bacterial culture (OD_{600}=0.8-1) in 1% of either Middlebrook 7H9 , Luria or Middlebrook 7H10 agar (See Appendix B) that had been cooled to 45°C and supplemented with 0.03% glucose, 1mM $CaCl_2$, 1mM $MgCl_2$ and 0.2% glycerol. A volume of 20ml of the agar was then poured into 90mm Petri-dishes and allowed to solidify, and stored at 4ºC until required. Culture filtrates containing potential phage (100µl) were spread plated on the indicator plates and allowed to dry. The plates were incubated at 37ºC and checked daily for plaque development.

2.3.6 Preparation of overlays for plaque assay: (Sambrook *et al.,* 1989)

The bottom agar consisting of 1% of Luria broth agar (See appendix B) or Middlebrook 7H10 agar (see Appendix B) was prepared and 5ml of this agar was added in 90mm Petri-dishes. For the top agar, 0.9% soft agar was prepared (see appendix B) and allowed to cool to 45°C at room temperature. A volume of 100µl either purified L5 or culture filtrate derived from *M. bovis* strains was added to 900µl of the indicator bacterial strain that had been grown to an optical density at 600nm of 0.8 to 1 (section 2.3.1), followed by addition of this mixture into 9ml of the soft agar in standard sterile glass test tube. The agar was mixed and poured onto the bottom agar and allowed to solidify at room temperature. The plates were then incubated at 37ºC, and monitored daily for plaque development.

2.3.7 Co-culturing experiment

A liquid culture of an L5 lysogen of *M. smegmatis* mc^2155 (OD_{600}= 1.31) was aliquoted into four equal volumes of 10ml each, in Falcon tubes (Lasec). Each culture was

inoculated with different concentrations of *M. smegmatis* mc^2155 wild type cells (OD_{600}=1.31) at ratio of lysogen to wild type cells: 1:0, 1:1, 1:10, and 1:100. The co-cultures were incubated at 37ºC, and sampled at different time intervals. The lysates were filtered with a 0.45µm non-pyrogenic hydrophilic disposable filter (see section 2.3.4) and monitored by standard plaque assay for plaque development as previously described (see section 2.3.5).

2.3.8 Epiflourescence Microscopy (EFM) to detect free phage particles (adapted from Noble and Fuhrman, 1998)

2.3.8.1 Preparation of culture filtrates

L5 lysogen and wild type *M. smegmatis* mc^2155, or *M. bovis* isolates phage filtrates were prepared by picking colonies that were freshly grown in Middlebrook 7H10 agar plates (see Appendix B) or the LJ slants (National Health Laboratory services (NHLS) for *M. bovis,* with a sterile inoculation loop and suspending this into 1ml phage buffer (Appendix B). Each of the bacterial suspensions was mixed by repeat pipetting. The bacterial cells were centrifuged at 13000rpm for 5 min, in a benchtop centrifuge followed by filtration through a 0.45µm disposable filter as described previously (section 2.3.4). The L5 phage titer from the lysogen was determined by plaque assay on *M. smegmatis* indicator plates as described in section 2.3.5.

2.3.8.2 Preparation of SYBR Green 1 Stain (Adapted from Noble and Fuhrman, 1998)

SYBR Green 1 is reported as a viable tool in the enumeration of virus particles by EFM. Double stranded DNA bacteriophage detection by this method is becoming more popular. The principle of this method lies on the ability of SYBR Green 1 to penetrate the phage capsid and intercalates on the nucleic acid. SYBR Green 1 has a proprietary formula and its manufacturer (Molecular Probes) does not report its molecular weight or concentration. The SYBR Green 1 (Molecular Probes) used for this study was diluted

1:10 of the supplied concentration in sterile de-ionized water with 0.2µm non-pyrogenic hydrophilic (Ministart) filtered deionised water, and stored at -20ºC. From the 10% stock solution a 2.5% working solution in sterile distilled water was prepared under subdued light (final dilution of $2.5x10^{-3}$)(An optical density of 0.42 at 494nm when the stock was diluted 1000, is how the concentrations were determined).

2.3.8.3 Specimen preparation (either purified L5, L5-lysogen or *M. bovis* culture filtrates)

For purified L5, 500µl was diluted to 5ml with phage buffer. For L5-lysogen or *M. bovis* contained on solid media, 500µl of culture filtrates (see preparation of culture filtrates in section 2.3.8.1) were added into Falcon tubes with 5ml phage buffer. The suspensions were each filtered separately onto a 0.02µm pore size Al_2O_3 Anodisc 25 membrane filters (Whatman) backed by 0.8µm nitrocellulose filter paper (Millipore) at approximately 20kPa vacuum. The Anodisc membranes were filtered to dryness, removed with forceps with vacuum still on.

2.3.8.4 Staining

Filtered material was stained by submerging the Anodisc filter in 100µl of SYBR Green 1 stain with sample-side facing up, as described in Noble and Furman (1998) for 15 min in the dark. After the staining period, the filter was picked up and any remaining moisture was then carefully wiped out by touching the back side of the membrane to a Kimwipe. The Anodisc filter was mounted sample up, on a glass slide. A few drops of mounting solution (50% Phosphate buffer saline (Appendix B), 50% glycerol, and 0.1% 2-phenylenediamine (Sigma Chem, made freshly from a freshly made 10% aqueous stock solution) was added onto the Anodisc filter and covered with a cover slip. The slides were observed under a Zeiss Axiocam EF microscope, under blue excitation at 1000× magnification. Representative fields were captured by a digital camera.

2.4 RESULTS

2.4.1 Isolation of spontaneously liberated phage

Before attempting to isolate spontaneously liberated phage directly from culture filtrates, we first attempted a non-chemical approach to see if it would lead to increased levels of spontaneously liberated phage in these culture filtrates.

2.4.1.1 Co-culturing a L5-*M. smegmatis* mc^{2}155 lysogen with *M. smegmatis* mc^{2}155

Spontaneous induction of prophages is generally low (10^{-2} to 10^{-5} phage per bacterium per generation – depending on the particular phage-host system) (Chibani-Chennoufi, 2004). In lysogenic cultures phage titers typically range between 10^4 and 10^5 pfu/ml in cultures where bacterial counts are in the order of 10^9 cfu/ml. This demonstrates a very small fraction of cells undergoing lysis, and this has no consequence to the majority of population that is immune to super infection (Bossi *et al.,* 2003). The situation has been shown to change if the population is not clonal and contains bacteria from a non-lysogenic strain (Bossi *et al.,* 2003). They showed that co-cultures of certain *Salmonella* strains carrying or lacking specific prophages undergo a swift composition changes as a result of phage mediated killing bacteria and lysogenic conversion of survivors. What happens is, i) Prophages are spontaneously induced in only a few lysogenic cell, ii) this enhances the competitive fitness of the lysogen population as a whole, setting a selection regime that forces maintenance and spread of viral DNA (Bossi *et al,* 2003), one of the byproducts being an elevation of free phage in the culture medium, which therefore offers itself as a potential method to amplify potentially low levels of phages in culture media.

In an attempt to see if this same approach may lead to a similar elevation of free phage in mycobacteria, and therefore may prove to be an effective method to increase the levels of free phage in the potentially lysogenic *M. bovis* outbreak strains we tested it with a known lysogen. An L5-*M. smegmatis* mc^{2}155 lysogen culture was co-cultured with its

non-lysogenic derivative at different ratios and tested for the levels of phage release by plaque assay.

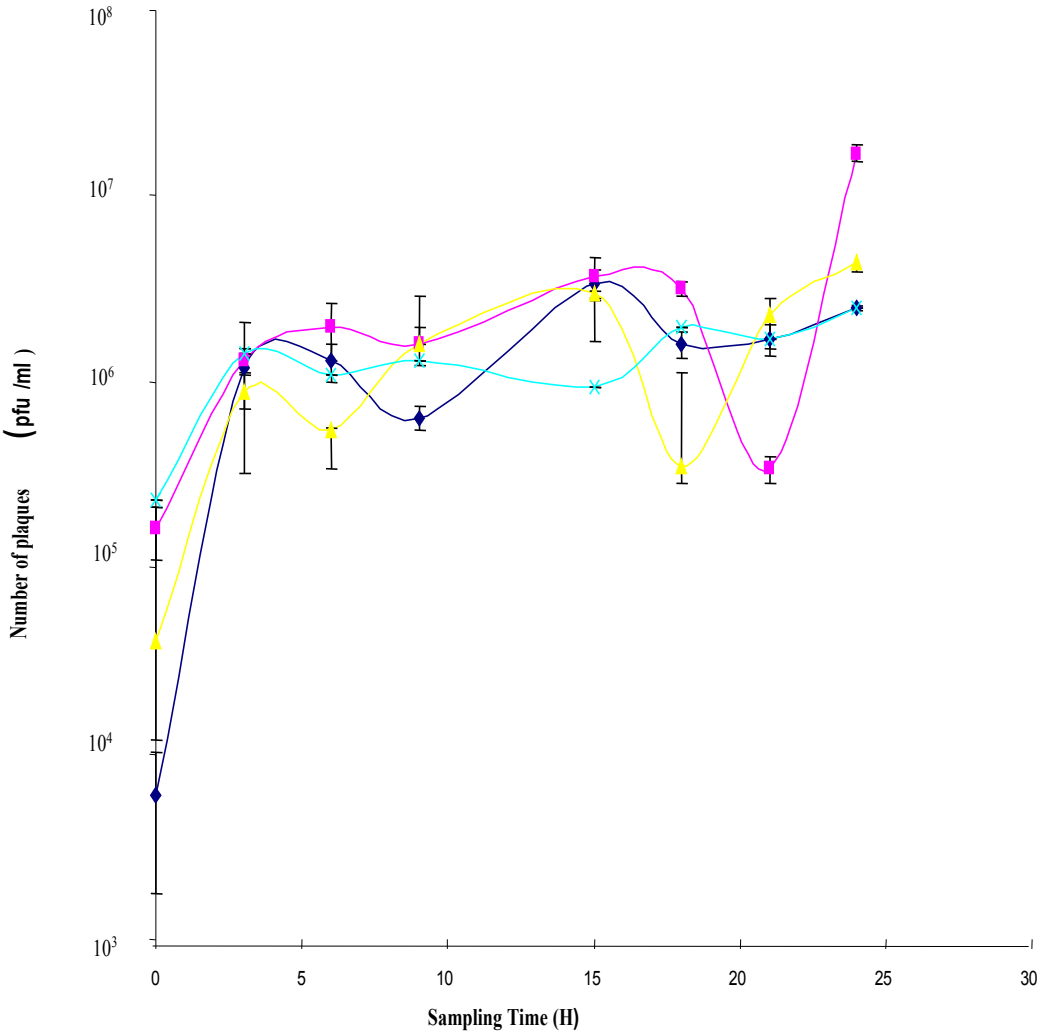

Fig 2: Co-infection experiment showing the levels of phage released by the L5-*M. smegmatis* culture when co-incubated with its non lysogenic counterpart. Plaques were obtained from plating duplicate samples from each co-incubation at different ratios of lysogen : non-lysogen. The y-error bars represent the standard errors obtained from three experiments. The different co-culture ratios are represented as ■ = 1lys: 1non-lys, △ = 1lys: 10non-lsy ✦ =1lys: 100non-lys, ◆ = no non lys. Lys= lysogen, non-lys=non lysogenic cells.

As can be seen in Fig 2, there is an initial rise in phage release in all co-cultures, followed by a drop after ~3 hours. Significant increases in phage release at the l: 1 and 1:10 co-infection cultures after 20 hours and 17 hours respectively were observed. As can be seen in Fig. 2, and contrary to what was observed by Bossi *et al,* (2003), co-culturing of L5-lysogen and non-lysogenic culture does not enhance the levels of spontaneously induced L5.

We therefore did not explore this approach further as a method to isolate phages from the *M. bovis* isolates.

2.4.1.2 Screening of *M. bovis* isolates for spontaneous phage induction using plaque assay

Since the previous approaches did not significantly suggest that we would be able to increase our yield of spontaneously liberated phage by co-incubation with potentially sensitive hosts, we decided to directly screen for induced phage. In this assay we screened for the presence of spontaneously inducible prophages from different *M. bovis* isolates. Filtrates from these isolates were plated on different bacterial indicator plates, and/or overlays and assayed for plaques. We reasoned that using different potentially sensitive indicator strains would increase our likelihood of isolating phages. As can be seen from Table 3, no plaques that could be propagated on the indicator strains were isolated from 27 *M. bovis* isolates. Interestingly in some cases turbid plaques were obtained after 3 months incubation that could not be propagated after the third serial passaging on indicator plates (Fig. 3). The plaques appeared to be different in size. One isolate TB 4031, as seen in Fig 3, produced big plaques that were approximately 5mm in diameter with a titer of 10^2pfu/ml, compared to the other two isolates TB 2773 and TB 4328 (result not shown), which both produced plaques that were approximately 1.5mm in diameter, with titers of 10^3 pfu/ml each. These three isolates were all from bovine species, but from different origins, suggesting that they may be different isolates and perhaps the plaques were different anti-mycobacterial entities too. The non- reproducing nature of the plaques were not characteristic of phages, hence we thought, even though this is unequivocally proven, that they might be bacteriocins.

Table 3: Screening for spontaneously induced phages from *M. bovis* isolates by plaque assay on different mycobacterial indicators.

***M. bovis* isolate number**	Tissue origin and animal species	Animal origin	**Mycobacterial Indicator Strains**				**Plaque Amplification**
			M. smegmatis	*M. fortuitum*	*M. scrofulaceum*	*M. kansasii*	
TB 3984E	Lung-Bovine	Middleburg (SA)	-	-	ND	-	-
TB 4006	Retro-Bovine	Stellenbosch (SA)	-	-	ND	-	-
TB 4016	Lung+ Liver- Bovine	Middleburg (SA)	-	-	ND	-	-
TB 4023	Liver+ Lung- Bovine	Ladysmith (SA)	-	-	ND	-	-
TB 4025	Thorax- Buffalo	Hoedspruit (SA)	-	-	ND	-	-
TB 2332	Lung- Buffalo	KNP (SA)	-	-	ND	-	-
TB 2338A	Swabs-Buffalo	KNP (SA)	-	-	ND	-	-
TB 2338B	Swabs-Buffalo	KNP (SA)	-	-	ND	-	-
TB 2891H	No record		-	-	ND	-	-
TB 2630B	Troncantor Bone-Lion	KNP (SA)	-	-	-	-	-
TB 2682E	Lung-Lion	KNP (SA)	-	-	-	-	-
TB 2649C	Lung- Buffalo	KNP (SA)	-	-	-	-	-
TB 2773	Mediastinal lympnode- Bovine	East London (SA)	+ (3months)*(pfu/ml=10^3)	+ (3months) *(pfu/ml=10^3)	ND	-	-
TB 2804	Lympnodes -Warthog	Mauriedale (SA)	-	-	ND	ND	-
TB 2836B	Lung –Baboon	Komatiepoort	-	-	NP	ND	-
TB 2881H	No record		-	-	ND	ND	-
TB 2916H	No record		-	-	ND	-	-
TB 3150	No record		-	-	ND	-	-
TB3878	Lympnodes- Bovine	Vryheid (SA)	-	-	ND	-	-
TB 4031	Lympnodes- Bovine	Nelspruit (SA)	+ (3months)* (pfu/ml=10^1)	+ (3 months)* (pfu/ml=10^1)	ND	-	-
TB 4328	Lympnodes- Bovine	Nat. Dept of Agriculture (SA)	+ (3months)* (pfu/ml=10^3)	+ (3 months)* (pfu/ml=10^3)	ND	-	-
TB 1764	No record		-	-	ND	-	-
TB 4326	Lympnodes- Bovine	Nat Dept of Agriculture (SA)	-	-	ND	-	-
TB 4330	Lympnodes- Bovine	Nat Dept of Agriculture (SA)	-	-	ND	-	-
TB 4388	Lympnode-Buffalo	Malelane	-	-	ND	-	-
TB 4329	Lympnode- Bovine	Nat Dep of Agriculture (SA)	-	-	ND	-	-
TB 4043	Lung- Eland	KNP (SA)	-	-	ND	-	-

-=No plaques, ND= not done. -= no plaque amplification, + = plaques observed, *time of incubation of culture at which plaques were observed, retro= retropharyngeal

A B

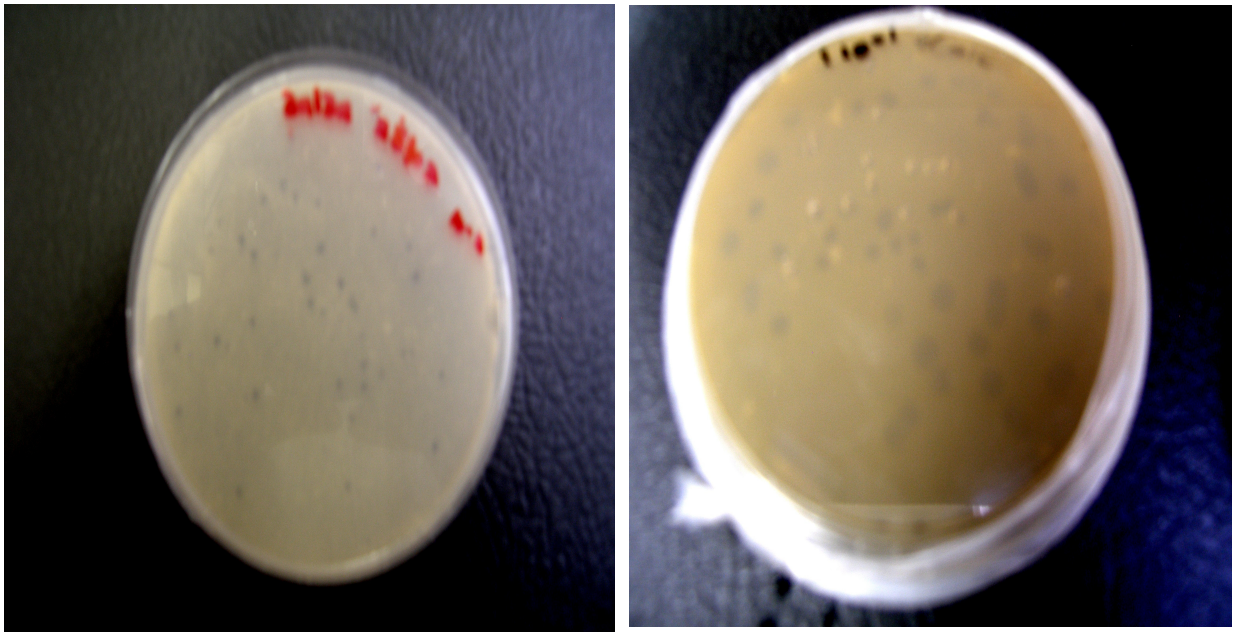

Fig 3: Plaques isolated from *M. bovis* isolates using plaque assay. **A**= Plaques from isolate TB4031, **B**=plaques from isolate TB2773.

Isolation of spontaneously inducible prophages liberated from *M. bovis* that could be propagated, using plaque assays with more than one sensitive strain was not achieved. This assay is a selective method that will only select for those phages that can infect the sensitive strains used. We reasoned that, if spontaneous phage induction from the isolates did occur, we might not have indicator strains that are sensitive to the released phages, hence we could have missed a certain proportion of the potential phages released. Therefore a non-selective approach was followed to investigate if any detectable phages are released from the *M. bovis* isolates.

2.4.1.3 Epiflourescence Microscopy (EFM) analysis

It is clear that the standard plaque assay methods for the isolation of spontaneously inducible phages are biased, which could result in a high proportion of associated phages being missed. We therefore attempted to identify if there were indeed any phages in the culture filtrates of the *M. bovis* isolates by non–selective EFM that will allow us to identify associated phages that have double-stranded DNA genomes (Vitzhum *et al.,* 1999; Zipper *et al.,* 2004).

Since this approach has not been tested on mycobacteriophages, we first tested to see whether we could detect the mycobacteriophage L5 using this approach. AL5-*M. smegmatis* mc^2155 lysogen culture grown on solid media was stained with SYBR Green I and tested for the presence of any spontaneously induced L5 phage particles. *M. smegmatis* mc^2155 wild type cells were also prepared for staining, to serve as a negative control. As can be seen from Fig. 4, the presence of free phage particles in L5 were observed in a single field by EFM as approximately ~110 bright spots in a titer of 10^2 pfu/ml(Fig 4c.), which were not seen in the negative control (Fig 4f). Our analysis therefore is that if EFM could detect 110 phage spots in 500µl=220spots/ml of phage suspension (see 2.3.8.3 in the methods section), in a titer of 10^2 pfu/ml as determined by plaque assay, therefore, a titer of 10^1pfu/ml, would be detected as 22 phage spots in a milliliter by EFM, and a titer of 10^0 pfu/ml would give 2.2 phage spots/ml. This implies that the EFM is so sensitive that it would detect two times the phage population detected by plaque assay.

Since EFM, contrary to the plaque assay, is not selective, i.e., it does not require a sensitive strain, which selects only phages that are capable of infecting only that strain, we reasoned that if there were any phages that might be released, we may be able to detect them, using EFM. Filtrates from solid cultures of *M. bovis* isolates were prepared, stained with SYBR Green 1, and viewed under EFM as described in the materials and methods (section 2.3.8). An *M. smegmatis* mc^2155 lysogen of L5 was employed as a

positive control (10^2pfu/ ml as determined by plaque assay), and a wild type solid culture of *M. smegmatis* mc^2155, was used as a negative control.

Out of sixteen samples tested we also included two out of three that formed non-reproducible plaques on *M. smegmatis* (See Table 3) of *M. bovis* tested for spontaneous phage induction, by EFM, a single viral-like spot, similar to the L5 control was observed in only one isolate: TB 4328. This isolate was also shown to form plaques with a titer of 10^3 pfu/ml on *M. smegmatis* and *M. fortuitum* (See table 3). Table 4 and figure 4a-f below shows the EFM results from the isolates tested.

Table 4: EFM results to screen *M. bovis* isolates for spontaneous phage induction in comparison to plaque assay results as previously determined.

Isolate number	Tissue origin and animal species	Animal origin	EFM result	Plaque assay result
TB 2773	Mediastal lympnodes-Bovine	East London	-	+ (3 months)*pfu/ml=10^3
TB 4328	Lymphnode- Bovine	Nat. dept of agriculture	±	+ (3 months)*pfu/ml=10^3
TB 3150	No record		-	-
TB 2916H	No record		-	-
TB 2804	Lympnode- Warthog	Maurieedale (SA)	-	-
TB 4036	Lympnodes- Bovine	Middleburg	-	ND
TB 4026	Mandible+ Retro- Bovine	Middleburg	-	ND
TB 4041	Lung- Beaver	KNP (SA)	-	ND
TB 4043	Lung- Eland	KNP (SA)	-	-
TB 5507D	Prescap- Impala	Malelani (SA)	-	ND
TB 5196A	Head- Buffalo	KNP (SA)	-	ND
TB 5119C	Head- Buffalo	KNP (SA)	-	ND
TB 5151A	Head- Buffalo	KNP (SA)	-	ND
TB 5082A	Lung- Lion	Hoedspruit (SA)	-	ND
TB 4931C	Left Retro- Buffalo	KNP (SA)	-	ND
TB 4898	No record		-	ND

±, potential viral-like spot; -, no viral spot, or plaque observed; +, plaques observed; retro, retropharyngeal; prescap, prescapular; *, refers to time following inoculation where plaque was observed.

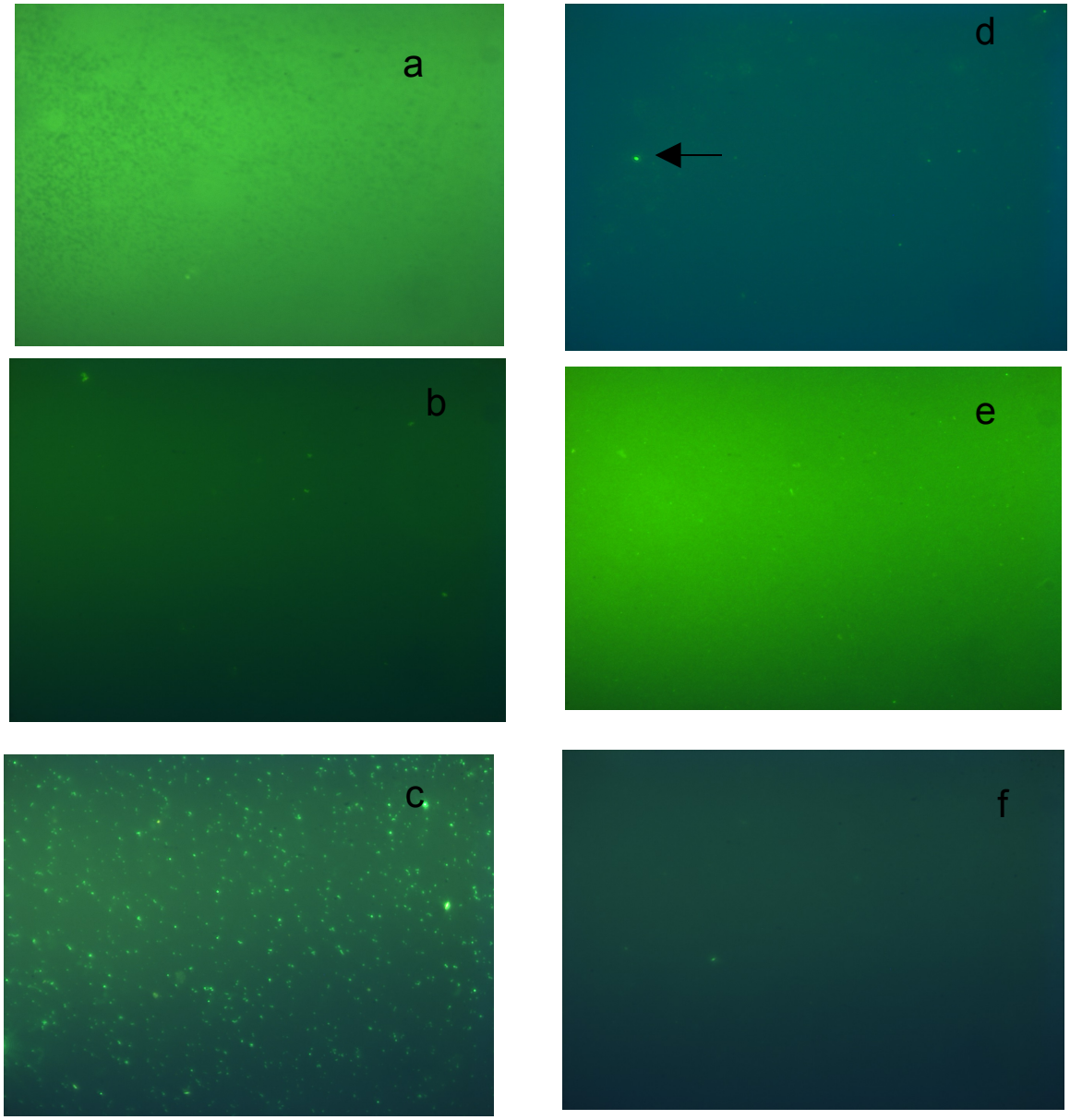

Fig 4: EFM of *M. bovis* culture filtrates. **a**= isolate TB4812, **b**=isolate TB 4931, **c**= L5 lysogen, **d**= isolate TB 4328,(→) points at a phage-like spot, **e**= isolate TB5082; **f**= *M. smegmatis* mc^2155 wild type.

Based on these results, spontaneous prophage induction from these *M. bovis*, is either very rare, or occurs below the levels of detection of our EFM assay. We reasoned that if we could find any inducible prophages in *M. bovis,* we may still be able to explore the presence of prophages that may be cryptic or simply undetectably low by using a prophage-like DNA sequences in the genomes of our *M. bovis* isolates.

It is clear that EFM is a feasible microscopic technique for screening for the presence of spontaneously induced phages in solid lysogenic cultures. This technique required a phage titer of as little as 10^2 pfu/ml (as determined by plaque assay), in order to detect individual phage in a single field. EFM can be used for direct screening and enumeration of phage particles in solid lysogenic cultures.

2.5 DISCUSSION

In this chapter we attempted to test whether we could increase the detectable levels of spontaneously liberated phage by co-incubating a lysogenic culture in the presence of a non-lysogenic culture. We found no significant difference after 24 hours co-incubation on the phage levels, even when high ratios of non-lysogen to lysogen were included. The phages, irrespective of the presence of a non-lysogen, increase in the initial stages of the incubation and reached a peak after approximately 3 hours. The lack of any difference in behavior from the non-co-incubation control was not expected since even when the ratio of the non-lysogenic cells to lysogens is high, there should be enough host cells for the phage particles to infect, which should then lead to relatively more cells being induced, as was observed by (Bossi *et al.,* 2003). We therefore expected the rate of phage release in co-cultures with higher ratio of host to lysogenic cells to be above that of the lower co-culture ratio. One possible explanation for the results obtained is that since L5 forms very stable lysogens with *M. smegmatis* cells (Snapper *et al.,* 1988; Lee *et al.,* 1991) only a small fraction of the newly formed lysogens undergo lysis.

Spontaneously induced phages from *M. bovis* isolates that could be propagated for further analysis could not be isolated by plaque assay methods. The possible reasons for this failure could be the following: i) Even though we used more than one indicator strain these cells may not have been sensitive for these phages, or ii) the *M. bovis* that we screened contained prophages that were not spontaneously inducible . One of the reasons for our inability to isolate phages from these isolates is due to the host specificity of bacteriophages (Mediavilla *et al.,* 2000; Hendrix, 2002). For example mycobacteriophage Bxb1 infects *M. smegmatis* but does not form plaques on slowly growing strains, (Mediavilla *et al.,* 2000). Other mycobacteriophages like L5, TM4, and D29, however have a broad host range ranging from slow growers and fast growers. Fullner and Hatfull (1997) have shown that phage L5 infection of slow growing *Mycobacterium bovis* BCG required increased levels of calcium ions, which are the conditions that differ from those required from infection of *M. smegmatis* by L5, and *M. bovis* BCG by phage D29. This difference in host specificity reflects the use of different receptor sites on the cell surface (Mediavilla *et al.,* 2000). It is clearly evident that the sensitivity of the host is a major

factor since Chen *et al.,* (2001); and Lu *et al.,* (2001) reported titers of usually 10^4-10^8 bacteriophage-like particles in marine ecosystems, as judged by either electron microscopy or fluorescence microscopy, and only a very small fraction (<1%) could be detected by plaque assay. Some studies revealed average bacteriophages concentration of $1.5x10^8$ particles per gram in some soils judged by microscopy and none of these visible reproduced after extraction into liquid medium and inoculation into liquid cultures (Ashelford *et al.,* 2003; and Williamson *et al.,* 2003). Phages of mycobacteria encounter a cell wall that is rich in unusual lipids and sugar containing components which form a formidable barrier which must be passed to gain access to the cell membrane (Barsom and Hatfull, 1996). Phage resistance phenotype of *M. smegmatis* to phages L5 and D29, were reported to result from and elevated expression of a multicopy phage resistance gene (*mpr)* whose product may alter the structure of the host cell wall or membrane thereby inhibiting effective injection of the phage DNA (Barsom and Hatfull, 1996).

Three isolates namely TB2773, TB4328, and TB4031 produced plaques with different morphologies on both *M. smegmatis* and *M. fortuitum* that could not be reproduced by amplification by serial passage. As can be seen in Table 3, the three isolates that produced plaques originated from bovine isolates from different geographic origins. The difference in plaque morphology may suggest that the causative agent may differ. These plaques were thought, from their antimicrobial characteristic and non-reproducing property, they might represent bacteriocins (Bradley *et al.,* 1967, Jack *et al.,* 1995) Bacteriocins are antimicrobacterial peptides produced by bacteria that inhibit the growth of the related species or even different strains of the same species (Saito *et al.,* 1979). Like bacteriophages, bacteriocins form plaques on bacterial lawns of their hosts, however unlike phages; the bacteriocin plaques are not reproducible. (Bradley *et al.,* 1967). Mycobacteriocins (bacteriocins of mycobacteria) have been reported in both the slow growing and fast growing mycobacteria. *Smegmaticin* (bacteriocin produced by *M. smegatis*), mycobacteriocin from *M. fortuitum* ATCC 6841/23012*, M. parafortuitum* ATCC 19648, *M. chelonei, M. phlei, M. chitinae* strains have been reported to form plaques on *M. diernhorefi.* (Saito *et al.,* 1983). Anti-*M. tuberculosis* bacteriocins have also been reported (Vasily *et al.,* 2007).

Since we failed to isolate plaques that could be propagated we explored the possibility that phage(s) may be released from *M. bovis* isolates, but that our indicator cells were simply insensitive to these. The sensitivity of the method was estimated by comparing the L5 phage titers as determined by EFM and plaque assay. When a titer of 10^2pfu/ml (as determined by plaque assay) was analysed by EFM, results showed 220 particles per milliliter of phage suspension in a representative field. We can therefore estimate that 10^0 pfu/ml should be detectable by EFM. Isolate TB4328 which formed plaques with a titer of 10^3 pfu/ml on *M. smegmatis* and *M. fortuitum* produced a single spot using EFM. Even though the EFM and the plaque assay results from this isolate could not be really comparable since the conditions under which these two methods were explored were different (with EFM a solid culture suspended in phage buffer was used, and with plaque assay, a liquid culture in Middlebrook 7H9 medium), however if we were to compare EFM and plaque assay, based on the estimated sensitivity of EFM and the titer of the plaques we observed from isolate TB4328, we would expect to see about 2000 particles from this isolate under EFM. Then we would conclude that the viral like spot observed in from this isolate represents an artifact. The rest of the other isolates screened showed no spontaneous phage induction (as determined by EFM). The fact that we only screened cultures from solid media at only one time point might be a limiting factor to our assay.

2.6 CONCLUSION

We were unable to isolate phage (plaque assay) or conclusively identify spontaneously release phage by non-selective EFM from *M. bovis* isolates. Even though we cannot conclude that these isolates did not release any phages, no reproducible plaques were produced on up to five different indicator plates. However three isolates from bovine species from different geographic origins produced different plaque forming units that could not be propagated for further analysis because of their non-reproducing characteristic. These were thought to be bacteriocins, since they could not propagate further on the indicator strains used. In addition, from EFM results which would detect any free phage including those that could not be detected by plaque assay, we can tentatively conclude that these *M. bovis* isolates lack prophages that are capable of producing phage particles. The presence of a phage like spot determined by EFM from one isolate was not enough to conclude that there was phage released, without any supporting evidence like TEM and viral DNA analysis. In addition the relative sensitivity of the method, as described by Chen *et al.,* 2001; Lu *et al.,* 2001 including the estimated sensitivity results in this study when L5 was used, also gave doubts that this phage –like spot was a phage particle. Our conclusion hence, taking into consideration the number of *M. bovis* isolates tested for spontaneous phage induction, the number of indicator strains used to isolate these phages, is that: spontaneous phage induction from this bacillus has proven to be very rare.

CHAPTER THREE- A GENOMICS SCREEN FOR PROPHAGES IN *M. bovis* ISOLATES

3.1 INTRODUCTION

Attempts to isolate and propagate phages from *M. bovis* isolates by spontaneous induction failed. Possible reasons for this are that this bacillus might contain cryptic prophages that are not capable of spontaneous excision from the bacterial cells; the indicator strains used were not sensitive to the phages that might have been released; the phages were very poorly induced.

Prophages can be available in many forms ranging from inducible phages, to cryptic prophages showing deletions, insertions and rearrangements to prophage remnants that have lost most of their phage genomes (Canchaya *et al.,* 2003). Only a few prophage genes are sufficiently conserved and distinct from bacterial genes, to serve as markers for prophage sequences. Hence unambiguous detection of cryptic prophages is difficult as they may be devoid of any cornerstone genes (Srividhya *et al.,* 2006). However, integrases are reported to be useful markers for identifying mobile DNA elements such as prophages in bacterial genomes, and NCBI BLAST searches may be used to detect integrase genes. However, it can be problematic to use an integrase as a prophage signature, as it is not clear as to what qualifies an integrase as prophage related, as there are many of these in bacterial genomes with diverse functions (Canchaya *et al.,* 2003). The integrase activity allows the insertion of open reading frames in the form of circular cassettes at the recombination site. Integrase family members have the distinctive ability to carry out a complete site-specific recombination reaction between two DNAs in the absence of high energy cofactors (Kwon *et al.,* 1997)

Other investigators have applied a prophage identification approach based on the codon usage analysis (Moszer *et al.,* 1999). This analysis however can be problematic as it cannot clearly differentiate between prophage sequences and other horizontally acquired elements (Canchaya *et al.,* 2003). Our approach to identify prophage sequences in this

bacillus was to look for genes encoding for repressors as these are also a hallmark feature of temperate phages and this afforded us a novel opportunity to see if we could use repressor sequences to screen for prophages. Repressor proteins function in the regulation of lysogeny maintenance and super infection immunity in diverse temperate phages (Scott *et al.,* 1978; Nesper *et al.,* 1999; Jain and Hatfull, 2000). Out of 30 sequenced mycobacteriophages, repressor sequences are reported in two mycobacteriophages in the NCBI database, namely, L5: gp71 and L1: *cIts*391 (www.ncbi.nlm.nih.gov), and in the literature in phage Bxb1: gp69 (Jain and Hatfull, 2000), Ms6:gp 6, Bxz2 gp: 74 (Sau *et al.,* 2003), phage Ch12: gp80 (Gomathi *et al.,* 2007). Phage D29 a lytic phage that shares about 80% of its sequences with L5 is believed to have lost its repressor functions (Ford *et al.,* 1998; Hendrix *et al.,* 1998), which is evidenced as a deletion of about half of gene 71 in the phage D29 genome when compared to the L5 genome (Ford *et al.,* 1998). The observation that phage D29 does not have an intact gp71 thus explains its lytic properties.

Mycobacteriophage L5 is well characterized at the molecular level (Fullner and Hatfull, 1997). It is a double stranded tailed phage that contains a linear dsDNA genome of 52297bp for which the entire DNA sequence is known. Its repressor protein (GP71) consists of 183-amino acids (Jain and Hatfull, 2000) and required for maintenance of lysogeny and is sufficient to confer super-infection. L5 together with seven other mycobacteriophages formed the basis for us to explore the use of repressor sequences as prophage signatures in mycobacteria. In this chapter *M. bovis* isolates were screened for prophages using mycobacteriophage repressor-like sequences to screen for these prophages in an approach using guessmer primers.

3.2 AIMS AND OBJECTIVES

Since temperate phages contain repressor proteins, we attempted to look for conserved sequences within the different mycobacteriophage repressor gene sequences in order to use them to design guessmer primers to screen *M. bovis* isolates for the related sequences. Our objectives therefore were:

- Design guessmer PCR primers based on conserved sequences in putative mycobacteriophage repressors.
- Optimize guessmer PCR conditions using a L5 -*M. smegmatis* mc^2155 lysogen.
- Screen *M. bovis* isolates for conserved sequences by PCR using guessmer primers
- Characterize the sequences

3.3 METHODS

3.3.1 Database searches

Homolog search for L5 GP71: Using a well characterized phage (L5) repressor protein (GP71) sequence retrieved from NCBI database (Accession No: Q05286), an NCBI BLASTP search which searches protein database using a protein query, was done to search for homologs. The identified sequences were all aligned with L5 by a local DNAMAN program using ClustalW algorithm to identify conserved sequences. The nucleotide sequences were obtained using the protein sequences to query the NCBI databases with the TBLASTN algorithm which searches translated nucleotide sequences. This was followed by retrieval of the gene sequences of the different mycobacteriophages, and these were aligned to facilitate design of the guessmer primers as described in the results.

Guessmer PCR products homolog search: An NCBI BLASTX search, which searches a protein database using translated query, was done to search for similar sequences, or protein homologs, using an e-value (a measure of the reliability of the score which measure the similarities of the queries to the obtained sequences) cut-off of 0.0001. The complete protein sequences of these homologs were retrieved from the NCBI database.

Multiple sequence alignments: The DNA sequences from the guessmer PCR products were translated using software contained within the DNAMAN suite of programs and aligned by ClustalW.

3.3.2 PCR

Table 5 below shows the primer sequences (synthesized by Inqaba Biotech) used in this study.

Table 5: List of Oligonucleotide primers sequences used for PCR.

Primer pair	Oligo Sequences	SOURCE
Guessmers		This study
RLRgp71	5'MGVCTNMGDGATCAYGGCGARTAC,	
DLLgp71	3'TTCGTTSACYCKGATSARBAGRTC	
Hsp65		(Telenti *et al.,* 1993)
Hsp65 For	5'ACCAACGATGGTGTGTCCAT,	
Hsp65 Rev	3'CTTGTCGAACCGCATACCCT	

3 The symbols in the guessmers represent: Y=pyrimidines (C/T), R=purines (A/G), N= C/G/A/T, M=A/C, A = A/G/T, V=A/G/C, D=A/G/T, S= G/C, K=G/T, B= C/G/T.

3.3.2.1 Template preparation

The *M. bovis* DNA templates were supplied by the ARC (OVI). They were prepared by suspending a colony grown in LJ slants in 100µl PCR grade water (Adcock Ingram). The suspensions were then boiled for 25 minutes, allowed to cool followed by centrifugation on a bench top Minispin centrifuge for 5 min at 12000rpm and stored at -20ºC until required.

The L5-*M smegmatis* mc^2155 lysogen, and *M. smegmatis* mc^2155 DNA templates were prepared by suspending a colony grown in Middlebrook 7H10 plates (see Appendix B), in 100µl PCR grade water (Adcock Ingram). The suspensions were boiled for 5 min., allowed to cool followed by centrifugation on a bench top Minispin centrifuge for 5 min at 12000rpm and stored at -20ºC until required.

3.3.2.2 PCR conditions

Guessmer PCR: The 50µl reaction mixture contained each a pair of guessmer primers used at a final concentration of 4µM each, 1 x NH_4 buffer [16mM NH_4, 67mM Tris HCl (pH 8.3), 0.01% Tween-20], 2mM $MgCl_2$, 1.5 mM dNTP mix, 3.75 U Bioline BioTaq™ DNA polymerase, and 2µl of DNA templates, which was prepared as described in section 3.3.2.1). The PCR reaction was carried out in a PCR Express (Omni Gene) thermocycler as follows: (denaturation: 95ºC, 45 s; annealing: 30 s, 55 ºC; elongation: 72 ºC, 30 s for 40 cycles). The optimum annealing temperature (55 ºC) of the guessmers was determined by gradient PCR.

Hsp 65 PCR: Two microlitres of cell lysate, which was prepared as described in section 3.3.2.1 was added to each reaction tube. The composition of the PCR mixture (50µl) was 2 µM (each) primer, 1 x NH_4 buffer [16mM NH_4, 67mM Tris HCl (pH 8.3), 0.01% Tween-20], 2mM $MgCl_2$, 1.5 mM dNTP mix and 3.75U Bioline BioTaq™ DNA polymerase. The PCR reaction was carried in a PCR Express (Omi Gene) thermocycler as follows: (denaturation: 95ºC, 45 s; annealing: 30 s, 59 ºC; elongation: 72 ºC, 30 s for 40 cycles).

3.3.2.3 Agarose gel electrophoresis

DNA samples were separated by electrophoresis in 1×TAE (see Appendix B) on a 1.5% agarose gel at 90V. The resulting gels were photographed using a gel doc system (BioDoc-It ™ System)

3.3.3 DNA fragment gel extraction and purification

Appropriate DNA bands were purified from the agarose gel using GFX PCR DNA and Gel band purification Kit (Amersham). The purification was done according to the manufacturer's specifications. Briefly the gel encompassing the DNA band was excised and weighed. A capture buffer (acetate and chaotrope, concentrations not given by the

manufacturer) was then added and mixed by voterxing. The mixture was incubated at 60ºC until the agarose was dissolved and then briefly centrifuged at 13000rpm for 30 s in a benchtop micro-centrifuge to diffuse DNA sample from the agarose. The sample was then put in a resin-based column that binds DNA and then centrifuged as described previously. The unbound material was then discarded and the column washed with a buffer [10mM Tris- HCl (pH 8.0); 1mM EDTA] and centrifuged as described previously. The DNA was eluted from the column with 20µl of sterile distilled water (sdH_2O). Recovery and yield of DNA was confirmed by agarose gel electrophoresis as described in section 3.3.2.3.

3.3.4 Cloning of the PCR products

The PCR products were cloned into pGEM T Easy (Promega) vector to enable the use of the primer sites situated on the flanks of the vector for sequencing (Appendix C). pGEM T Easy vector contains 3'T overhangs at the insertion site providing a compatible overhang for PCR products generated by thermostable polymerases, like the *Taq* polymerase. It also contains the multiple restriction sites within the multiple cloning regions (See Appendix C). The clones were transformed into *E. coli* MM294 competent cells. The *E. coli* MM294 cells were obtained from Prof Dabbs' lab from the University of Witwatersrand.

3.3.4.1 Preparation of Competent *E. coli* MM294 Cells

Chemically competent cells of *E. coli* MM294 were freshly prepared by growing a 5ml pre-culture. This was prepared by inoculating 5ml LB with a single colony from a LB agar plate (see composition in Appendix B) and incubating at 37ºC overnight to allow the culture to reach stationary phase. A secondary culture was prepared by inoculating 20ml of a pre-warmed LB containing 0.5% glucose with a 200µl of the pre-culture. The culture was grown with aeration for ~ 2 hours at 37ºC till an optical density of ~0.4 at 540nm was reached. The cells were then incubated on ice for 5 min. followed by centrifugation for 5 min. at 4 ºC, at 10,000 rpm. The supernatant was then discarded and

the cells resuspended in half the volume (10ml) of a transformation buffer (1.5mM $CaCl_2.2H_2O$ and 10mM Tris-HCl) and left on ice for at least 15 min. The suspension was then centrifuged at 4ºC, at 10,000 rpm for 5 min. and again, the supernatant was discarded. The cells were resuspended in 1.3ml of transformation buffer and left on ice for 2 hours prior to transformation.

3.3.4.2 DNA ligations

DNA ligations were prepared as stipulated in the manufacturer's manual (Promega Corporation). Briefly, 5µl of 2×ligation buffer; 1µl pGEM-T Easy vector (50ng); PCR product (~50ng-80ng); 1µl T4 DNA ligase (3units/µl); deionised water to a final reaction volume of 10µl were mixed and incubated at room temperature for 1h followed by overnight storage at -20ºC. The ligation reactions were them placed on ice for 10 min to equilibrate the temperature with that of the competent cells.

3.3.4.3 Transformation conditions

To each ligation reaction, 50µl of competent cells was added and mixed by gentle tapping. The mixtures were left on ice for 10min. They were then heat shocked for 90 sec at 44ºC. Aliquots of 0.5ml of LB were then added to each tube and incubated at 37ºC for 60min. to allow phenotypic expression of Amp^r. The cells were spread plated on ampicilin (100µg/ml) containing plates and incubated at 37 ºC for 24h to develop colonies.

3.3.5 Characterization of clones

The clones were characterized by: i) plasmid DNA extraction from the transformants to confirm the presence of vector; ii) digestion of the plasmids to release the insert DNA; iii) sequencing of insert DNA, and iv) analysis of the obtained sequences to determine their identity and localization in the *M. bovis* genome.

3.3.5.1 Plasmid DNA extraction from transformants (adapted from Sambrook *et al.*, 1989)

Colonies that grew on ampicilin containing plates were picked and each was grown in 5ml ampicilin (100µg/ml)-containing LB medium, at 37ºC overnight in a shaker at 120rpm. The following day, 1.5 ml of the culture was transferred to sterile eppendorf tube. The cells were pelleted by centrifugation in a bench top centrifuge at 12000rpm for 2 min. The supernatant was then discarded. Another 1.5 ml bacterial culture was added and centrifuged again to increase the yield of the plasmid DNA. The supernatant was again discarded and the pellet recentrifuged for 10s. The remaining medium was carefully removed and discarded. A volume of 80µl of Solution 1 [50mM glucose, 25mM Tris.Cl (pH 8.0), 10mM EDTA (pH 8.0)] was added and resuspended by pipetting. To this mixture, 160µl of freshly prepared Solution II [0.2N NaOH, 1% SDS] was added and mixed by inversion to lyse the bacterial cells and denature the proteins. The solution was kept on ice for 3min. following which 120µl of solution III [3M potassium acetate, glacial acetic acid (pH 4.8)] was added to precipitate the cell debris and chromosomal DNA. The solution was then mixed by vortexing, followed by a further incubation on ice for 10min. This was then centrifuged for 10min at 13000rpm at 4ºC to separate the precipitated cell debris and the nucleic acid. The DNA containing supernatant was transferred to a fresh sterile Eppendorf tube, and 1µl of a 10mg/ml RNAse A was added to eliminate RNA and incubated at 37ºC for 60min. The plasmid DNA was then precipitated by adding 450µl of isopropanol and centrifuged at 13000rpm for 10 min. at room temperature in a bench top centrifuge to pellet the plasmid DNA. The supernatant was discarded and the pellet washed with 1ml 70% ice-cold ethanol. The pellet was dried and resuspended in 20µl of distilled water, and stored at -20ºC.

3.3.5.2 Plasmid DNA digestion

Plasmid DNA (~500ng to 800ng) (concentration estimated by comparison of the DNA band intensities of the extracted plasmids with the control plasmid [puC18 whose concentration was provided in the manufactures manual (~0.5mg/ml)] was digested with

*Eco*R1 enzyme [(0.025units) (Fermentas)] to release the inserts from the plasmid vector (see vector map in Appendix C). The digestion reaction was carried out as follows: [5µl plasmid DNA (~50ng-80ng), 1.5µl *Eco*R1 buffer [50mM Tris HCl (pH 7.5), 10mM, $MgCl_2$, 100mM NaCl, 0.02% Triton X-100, and 0.1 mg/ml BSA], and 0.5µl *Eco*R1enzyme [0.025units (Fermentas)]. Each reaction mixture was incubated at 37ºC for 7 to 24h. The resulting digests were run on a 1.5% agarose gel in 1×TAE buffer at 90V as previously described (see section 3.3.2.3). The gels were photographed and the migration distance of the inserts measured to determine their sizes using a standard curve plotted for the molecular weight marker standards that were run together with the digests.

3.3.5.3 Sequencing and sequence analysis

The plasmid DNA samples were sent to Inqaba Biotechnologies for sequencing using automated sequencing. The sequencing primers used were M13 primers. The resulting sequences were downloaded from the chromatogram files using Chromas and a homolog search for similar sequences and multiple alignments of the sequences with the L5GP 71 were done as described in section 3.3.1.

3.4 RESULTS

3.4.1 Development of guessmer primers for detection of mycobacteriophage repressor like sequences in *M. bovis* isolates

Isolation of phages from *M. bovis* isolates by spontaneous induction was not achieved. We reasoned that the isolates may contain prophages that are not capable of spontaneous induction. We therefore used a genomics approach to screen for such prophages. Since temperate phages contain repressor proteins**,** fishing for repressor sequences as a way of screening the *M. bovis* isolates for prophages was justified. Using the phage L5 repressor gene 71, a comparative analysis of the repressor protein of other sequenced mycobacteriophages and L5 was done. NCBI BLASTP search revealed related and conserved sequences of the repressor protein at amino acid level with other sequenced mycobacteriophages, viz L5, Bxb1, Bxz2, Che12, L1, M6, Bethlehem, and U2 (see Table 6). Alignment of these repressor–like proteins using ClustalW showed at least 5 blocks of sequences carrying 4-8 identical amino acid residues (Fig. 5). These sequences were identified in eight out of thirty sequenced mycobacteriophages, and also one of these eight amino acid residues: RLRDHGEY is also conserved in lytic phage D29. Phage D29 is believed to have been derived from a temperate L5-like phage, where part of its repressor sequence is missing and this represents the essential difference between L5 and D29 (Ford *et al.,* 1998). Interestingly, as can be inferred from Table 6, all known temperate mycobacteriophages fall within this group of eight phages. The eight amino acid residue blocks- RLRDHGEY (L5 gp71 position 85-92/ block iii in Fig 5) and DLLIRVNE (L5 gp71 position 143-150/ block v in Fig 5**)** were selected for guessmer design, even though DLLIRVNE was not fully conserved in phage Bxz2 (block v; positions 114,115, and 119, Fig 5) since they represented the largest blocks of conservation.

Table 6: Mycobacteriophage with identical repressor–like proteins, their origin and function.

Mycobacteriophage	Gene product	Function of protein	e-value	Phage Lifestyle
L5	gp71	Phage repressor protein	$2e^{-97}$	Temperate
L1	*cI*ts391	Temperature sensitive repressor	$1e^{-96}$	Temperate
MS6	gp6	Phage repressor protein*	$2e^{-29}$	Temperate
U2	gp68	Unassigned	$2e^{-28}$	ND**
Che12	gp80	Phage repressor*	$4e^{-67}$	Temperate
Bxz2	gp74	Unassigned	$6e^{-26}$	Temperate
Bxb1	gp69	Phage repressor*	$2e^{-28}$	Temperate
Bethlehem	Gp77	Unassigned	$2e^{-28}$	ND**

***, Putative; **, not determined**

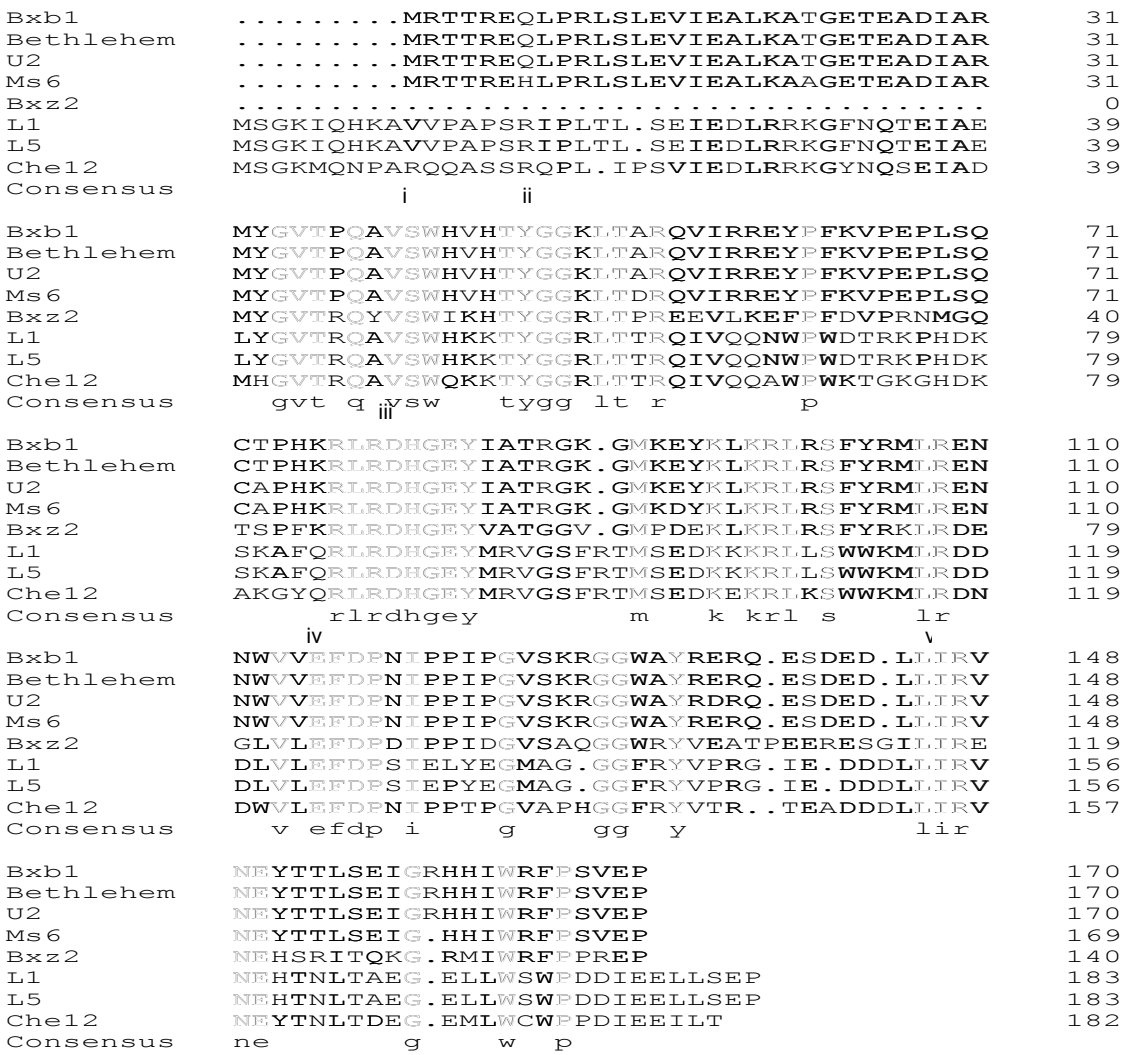

Fig 5: The alignment of the mycobacteriophage GP71 (L5) repressor-like amino acid sequences. The amino acid sequences of the repressor proteins of Bxb1, L1, L5, Che12, Ms6, Bethlehem and U2 were aligned by DNAMAN program (ClustalW). The blocks carrying identical, or nearly identical, amino acids are shaded in black. The different phages are on the left, and the numbers on the right of the figure represent the position of the amino acids within the repressor-like sequences. The numbers I, ii, iii, iv, v show blocks of four or more identical amino acids.

Table 7 shown below, represents the derived nucleotide sequences which was used to design the following guessmer sequences, Forward:
MGVCTNMGDATCAYGGCGARTAC and Reverse**:**
TTCGTTSACYCKGATSARBAGRTC based on the resulting DNA consensus sequences shown in Table 7.

Table 7. Nucleotide sequences derived from conserved amino acid sequences in mycobacteriophage repressor sequences.

phage and phage repressor-like protein	5' to 3' DNA sequence corresponding to RLRDHGEY amino acid sequence																							
L5*gp71**	A	**G**	G	C	**T**	C	C	G	G	G	**A**	T	C	**A**	C	**G**	**G**	C	G	A	G	**T**	**A**	**A**
Bxb1*gp69**	C	**G**	C	C	**T**	A	A	G	G	G	**A**	T	C	**A**	C	**G**	**G**	C	G	A	A	**T**	**A**	**C**
L1* *c1*ts391**	A	**G**	G	C	**T**	C	C	G	G	G	**A**	T	C	**A**	C	**G**	**G**	C	G	A	A	**T**	**A**	**C**
Bxz2*gp74**	A	**G**	A	C	**T**	T	C	G	T	G	**A**	C	C	**A**	C	**G**	**G**	G	G	A	G	**T**	**A**	**C**
Ms6*gp6**	C	**G**	A	C	**T**	G	A	G	G	G	**A**	T	C	**A**	T	**G**	**G**	C	G	A	A	**T**	**A**	**C**
U2*gp68**	C	**G**	A	C	**T**	G	A	G	G	G	**A**	T	C	**A**	T	**G**	**G**	C	G	A	A	**T**	**A**	**C**
Bethlehem*gp77**	C	**G**	A	C	**T**	G	A	G	A	G	**A**	T	C	**A**	C	**G**	**G**	C	G	A	A	**T**	**A**	**C**
Consensus***	A/C	**G**	A/C/G	C	**T**	A/C/G/T	A/C	G/T	A/G/T	G/C	**A**	C/T	C	**A**	C/T	**G**	**G**	C/G	G/A	A/T	A/G	**T**	**A**	**C**
	5' to 3' DNA sequence corresponding to DLLIRVNE amino acid sequence																							
L5*gp71**	**G**	**A**	T	**C**	**T**	C	C	**T**	G	**A**	**T**	**C**	C	**G**	G	**G**	**T**	G	**A**	**A**	**C**	**G**	**A**	G
Bxb1*gp69**	**G**	**A**	C	**C**	**T**	A	C	**T**	C	**A**	**T**	**C**	A	**G**	A	**G**	**T**	C	**A**	**A**	**C**	**G**	**A**	A
L1**c1*ts391**	**G**	**A**	T	**C**	**T**	C	C	**T**	G	**A**	**T**	**C**	C	**G**	G	**G**	**T**	G	**A**	**A**	**C**	**G**	**A**	G
D29*gp72**	**G**	**A**	C	**C**	**T**	G	T	**T**	G	**A**	**T**	**C**	C	**G**	G	**G**	**T**	G	**A**	**A**	**C**	**G**	**A**	G
MS6*gp6**	**G**	**A**	C	**C**	**T**	A	C	**T**	C	**A**	**T**	**C**	A	**G**	A	**G**	**T**	C	**A**	**A**	**C**	**G**	**A**	A
U2*gp68**	**G**	**A**	C	**C**	**T**	A	C	**T**	C	**A**	**T**	**C**	A	**G**	A	**G**	**T**	C	**A**	**A**	**C**	**G**	**A**	A
Bethlehem*gp77*	**G**	**A**	C	**C**	**T**	A	C	**T**	C	**A**	**T**	**C**	A	**G**	A	**G**	**T**	C	**A**	**A**	**C**	**G**	**A**	A
Consensus***	**G**	**A**	C/T	**C**	**T**	A/C/G	C/T	**T**	C/G	**A**	**T**	**C**	A/C	**G**	A/G	**G**	**T**	C/G	**A**	**A**	**C**	**G**	**A**	A/G

FORWARD PRIMER: RLRGP71 (24 mer)																							
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A/C	G	A/C/G	C/A	T	A/C/G/T	A/C	G/T	A/G/T	G/C	A	C/T	C/A	A	C/T	G	G	C/G	G/A	A/T	A/G	T	A	C

REVERSE PRIMER :DLLIGP71 (24 mer)																							
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
T/C	T	C	G	T	T	C/G	A	C	C/T	C	G/T	G	A	T	C/G	A	A/G	C/G/T	A	G	A/G	T	C

Identical nucleotide bases occurring at the same positions in the repressor-like sequences are indicated by bold letters, *, indicates the phages, **, the gene products , and * indicates the consensus arrived at by putting together the resulting different nucleotide bases that occur at the same positions in the sequences of the different phages.**

3.4.2 Amplification of L5 gp71 using guessmer primers, and PCR analysis

A L5-*M.smegmatis* mc^2155 DNA was used to evaluate the guessmer primers. As can be seen from Fig. 6, the PCR yielded a DNA band that appeared to be of the size anticipated for the L5 gp71 (222bp), and the water control did not amplify. A *M. smegmatis* mc^2155 wild type control was employed later when the *M. bovis* isolates were assessed. This control also did not amplify as can be seen in Fig 7b, lane 2. The PCR product from the L5-*M. smegmatis* mc^2155 was sequenced and BLAST analysis revealed it to be 100% identical to the region encompassing the conserved sequences in the gp71 L5 repressor sequence.

3.4.3 Screening of *M. bovis* isolates for prophages using guessmer primers

Since we were able to amplify the correct L5 gp71 sequence using the L5-*M.smegmatis* lysogen we then screened *M. bovis* isolates using the guessmer primers to evaluate the presence of potential prophages. Out of 45 *M. bovis* isolates screened for prophage repressor sequences, 9 produced amplification products (Fig 7a and 7b). As can be seen in Fig 7a, 7b and Table 8, the amplicons from each positive sample produced DNA bands of different sizes: ~1000bp, ~600bp, ~420bp, and ~220bp. Given that only 9 out of 45 isolates amplified, we investigated whether this was strain specific or whether it was due to insufficient DNA content in the other 36 samples that did not amplify. Therefore to confirm the quality and mycobacterial status of the *M. bovis* DNA templates that were screened for prophages by guessmer PCR, we used *hsp*65 genus-specific primers which is a genus-specific marker of mycobacteria (Telenti *et al.,* 1993). As can be seen from Fig. 8a and 8b, amplification using these primers resulted in DNA bands that appeared to be of the expected size of 439bp (gel a, lanes 3-8; gel b, lanes 2-9 and gel c, lanes 1-4, and 7-8) and were identical to the *M. smegmatis* positive control (gel b, lane 9). Importantly a control lacking added template failed to amplify (Fig 8a, lane 1).

All 9 of the guessmer positive templates also yielded positive *hsp65* amplicons (Fig 7 and 8; Table 8). Twenty isolates that were guessmer negative appeared to be *hsp*65 positive. We also noted that 11 isolates that did not amplify with the guessmers also did not amplify with the *hsp*65 (Table 8), and we reasoned that in these samples there was insufficient DNA of good quality. All guessmer positive products yielded a similar amplicon profile that importantly was missing from both the no template control (Fig 7b; lane 1) as well as the *M. smegmatis* mc^2155 control (Fig 7b; lane 2). This amplification profile is not common to all *M. bovis* isolates as not all *hsp65*-positive showed these amplification products, suggesting perhaps some strain specific activity.

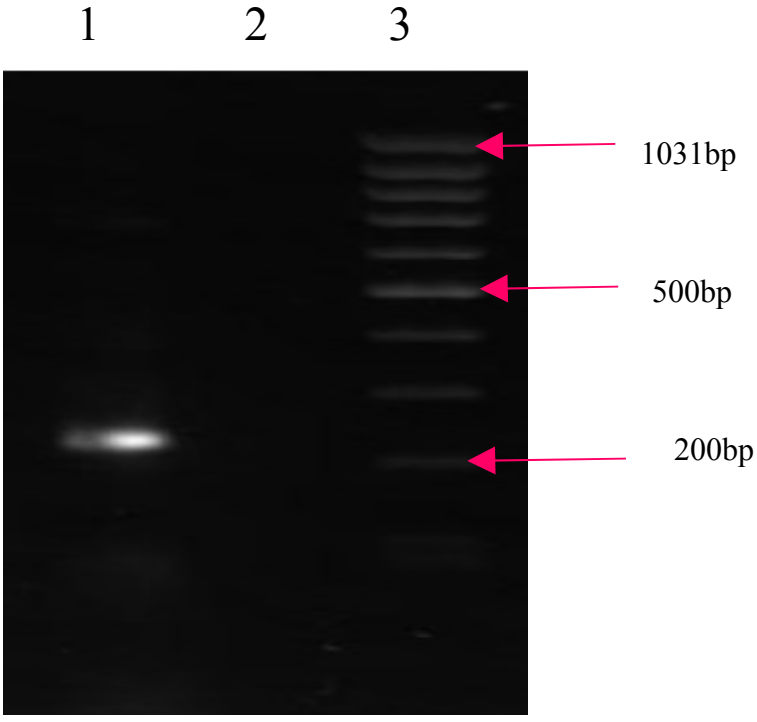

Fig 6: Amplification of the L5-GP71.PCR product of the L5-*Msmegmatis* lysogen .Lanes1=L5-lysogen,2=H_2O, 3=50bp O'Gene Ruler.

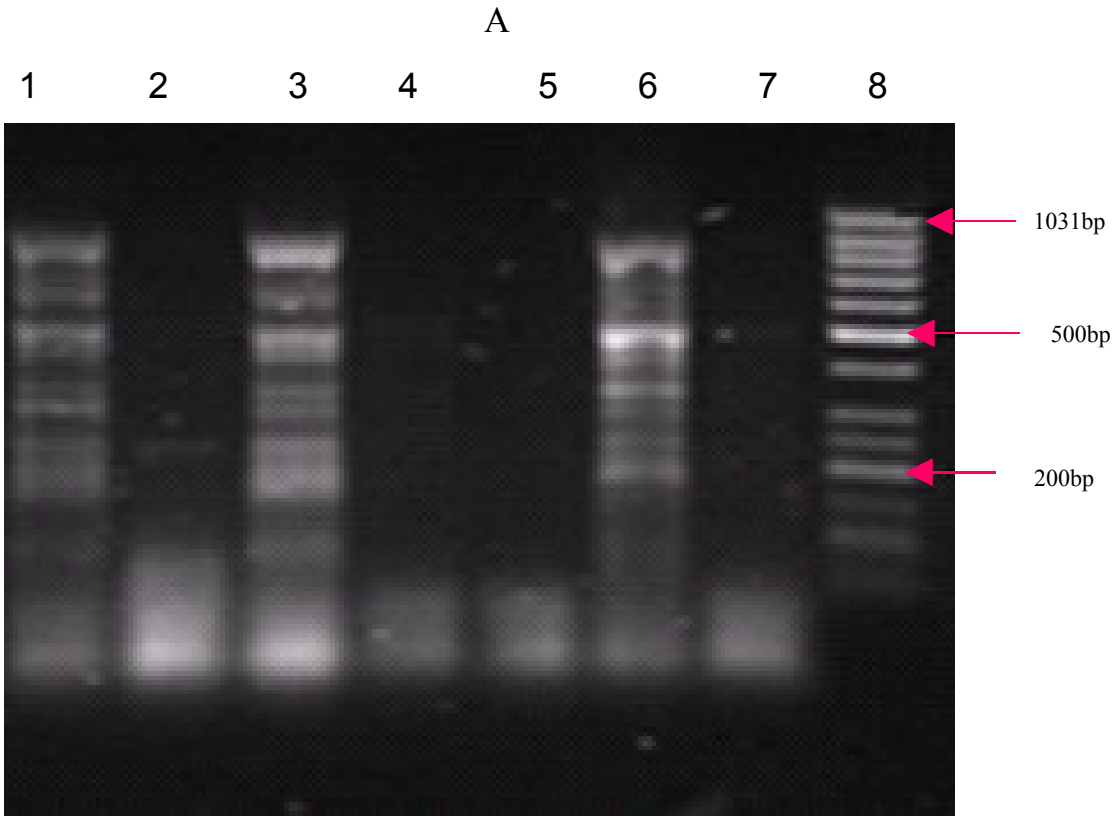

Fig 7 A: Screening of *M. bovis* isolates for cryptic prophages using guessmer PCR. PCR Products were ran on 1.5% TAE agarose gel. Lanes 1-7= isolates: TB5109B, TB1066+, TB4931C, TB5149E, TB4760C, TB5151A, TB4812C, 8=50bp O'range Ruler

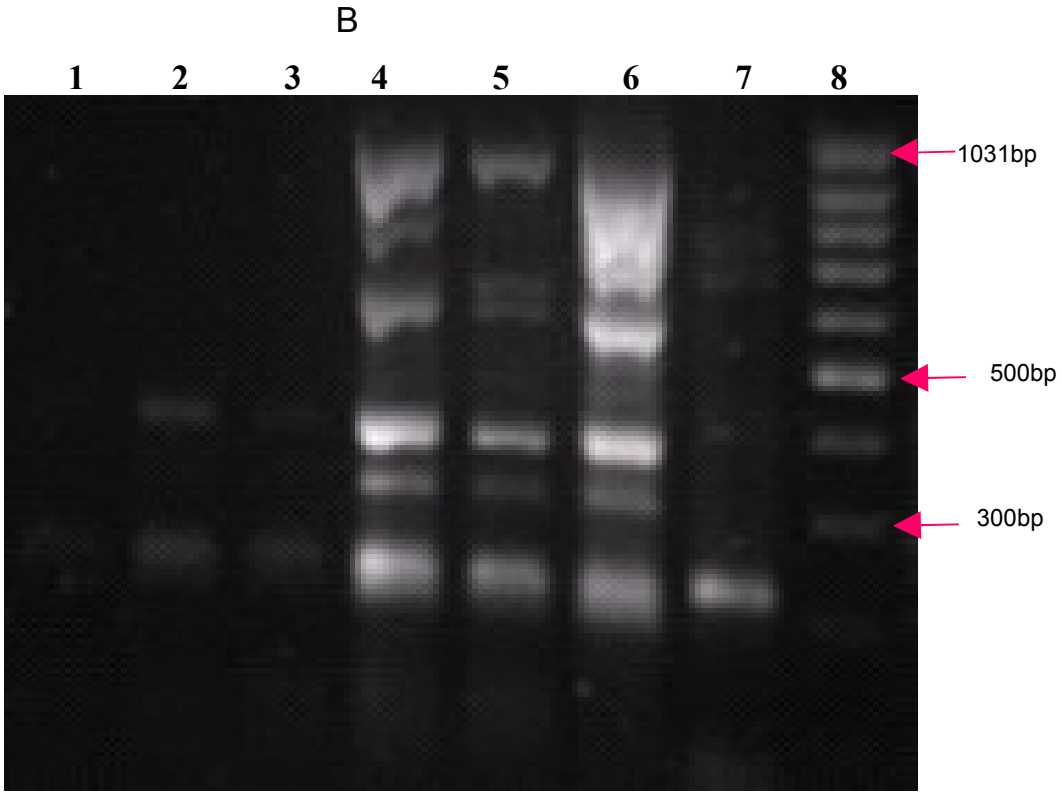

Fig 7B: Screening of *M. bovis* isolates for cryptic prophages using guessmer PCR. The PCR products were ran on 1.5% TAE agarose gel. Lanes 1-7= H_2O, *M. smegmatis*, TB4812C, TB4931C, TB5082, TB4850D, L5- lysogen, 8= 50bp O'range Ruler

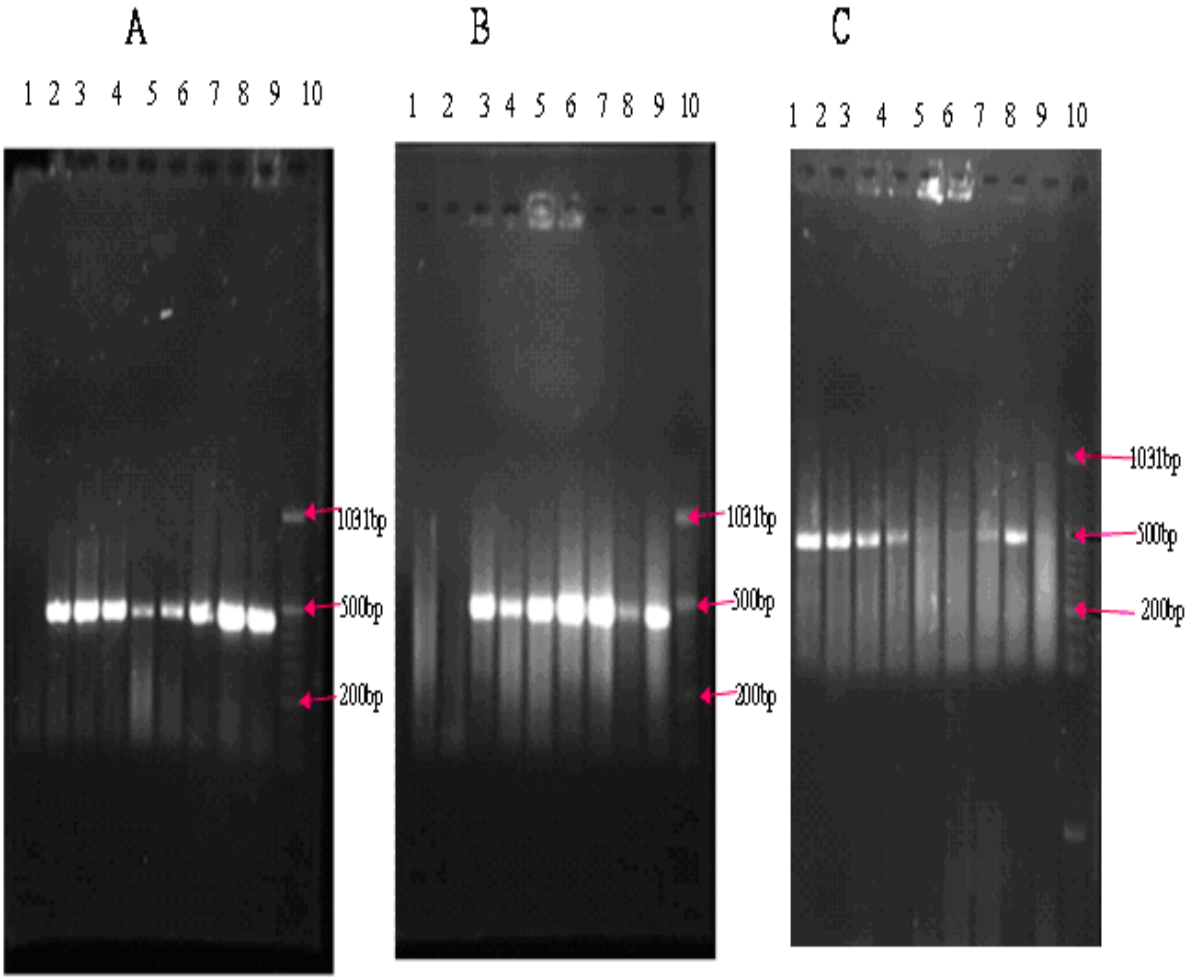

Fig8.:PCR based confirmation of the mycobacterial status of the *M. bovis* isolates using Hsp65 primers. PCR products were ran on a 1.5% agarose gel. **A,** Lane 1= H_2O, lanes 2-9=isolates: TB5150, TB5149D, TB5952A, TB3867A,Tb 5149E, TB1755C, TB 3569,TB4760C, Lane 10= O'range DNA Ladder. **B,** Lane 1=H_2O, Lanes 2-9=Isolates: TB499OC, TB4850D, TB4935, TB5149, TB5122, TB5751A, TB4931C, *M. smegmatis* mc^2155, lane 10= 50bp O'range DNA Ladder. **C,** Lanes 1-9= TB4812J, TB3107A, TB5234A, TB3869G, TB1158Mtb, TB1066+, TB3522, TB4992B, TB4675, Lane 10= 50bp O'range DNA Ladder.

Table 8: Summary of PCR based screening of *M. bovis* isolates for prophages, and *hsp65* mycobacterial confirmation of the isolates.

M.bovis isolate number	Tissue origin and animal species	Animal Origin	Guesmer PCR	Hsp 65 PCR
TB4850D	Thorax- Buffalo	KNP (SA)	+	+
TB4759C	Mesentric gland- Bovine	Ladysmith (SA)	-	+
TB5149D	Mandibles-Buffalo	KNP (SA)	+	+
TB4992B	Thorax-Buffalo	KNP (SA)	-	-
TB4675	Mesentric gland-Porcine	Ladysmith (SA)	-	-
TB4893A	Spleen- Monkey	Mauritius	-	+
TB4760C	Medistal -Bovine	Ladysmith (SA)	-	+
TB4812J	Axil-Buffalo	KNP (SA)	-	+
TB4804A	Left Retropharyngeal-Buffalo	KNP (SA)	-	+
TB4860F	Lung-Buffalo	KNP (SA)	-	-
TB4933A	Left Retropharyngeal- Buffalo	KNP (SA)	-	+
TB4804F	Thorax- Buffalo	KNP (SA)	-	-
TB4990C	Carcass- Buffalo	KNP (SA)	-	-
TB4816F	Thorax+ Lung- Buffalo	KNP (SA)	-	+
TB4812C	Left Tonsil- Buffalo	KNP (SA)	-	+
TB4862B	Right Retro-Buffalo	KNP (SA)	-	-
TB4779A	Lung+ Bronchi- Bovine	Ladysmith (SA)	-	-
TB4865A	Left Retropharyngeal- Buffalo	KNP (SA)	-	+
TB5149E	Lung - Buffalo	KNP (SA)	-	+
TB4931C	Left Retropharygeal-Buffalo	KNP (SA)	+	+
TB5149C	Bone Marrow-Buffalo	KNP (SA)	+	+
TB1066B+	No record		-	+
TB5151A	Head- Buffalo	KNP (SA)	+	+
TB5109B	Thorax- Buffalo	KNP (SA)	+	+
TB5082A	Lung- Lion	Hoedspruit (SA)	+	+
TB5150A	Head- Buffalo	KNP (SA)	-	-
TB1158Mtb	No record		-	-
TB1158Tfa	No record		-	-
TB3197	No record		+	+
TB3569G	No record		-	+
TB5123D	Left Retropharyngeal- Buffalo	KNP (SA)	+	+
TB4952C	Sternum+ Lung-Buffalo	KNP (SA)	-	+
TB3670	No record		-	+
TB5234B	Muscle- Lion	KNP (SA)	-	+
TB5122A	Head- Buffalo	KNP (SA)	-	+
TB4893D	Mesentric gland- Monkey	Mauritius	-	Nd
TB3869G	No record		-	+
TB1071B	No record		-	Nd
TB1755C	No record		-	+
TB3107A	No record		-	+
TB3522	No record		-	+
TB3534	No record		-	Nd
TB4935D	Right tonsil-Buffalo	KNP (SA)	-	+
TB4936A	Right Retropharyngeal- Buffalo	KNP (SA)	-	Nd
TB4975B	Spleen- Buffalo	Cascades (SA)	-	-

+= There was amplification, -= no amplification, Nd= PCR not done. KNP= Kruger National Park, SA= South Africa,

3.4.4 Cloning and sequencing of the PCR products

In order to determine if our guessmer PCR amplified DNA were of prophage origin, we determined the DNA sequences of some of the amplicons. We could not obtain readable sequence information by sequencing directly with the guessmer primers, even though this worked for PCR product derived from the L5-*M. smegmatis* lysogen (section 3.4.2). We decided therefore to first clone the PCR products from the *M. bovis* DNA to enable the use of the primer sites flanking the vector, for sequencing.

Since the DNA banding patterns of the *M. bovis* isolates tested for prophages appeared to be similar, only two samples, namely TB 4850D and TB5151 (Fig7 , gel B, lane 6 and Fig 7 gel A, lane6 respectively) were selected for cloning and subsequent sequencing of the cloned insert. Four DNA bands from sample TB4850D (DNA samples of approximately 1000bp, 600bp, 390bp and 220bp respectively), and one DNA band from TB5151 (approximately 420bp in size) were selected for cloning and were separately ligated into pGEM T- Easy vector as described in section 3.3.5.2. Transformants were selected and analysed from each ligation, and were digested with *Eco*R1 which should release the insert (see Appendix C) and puC18 plasmid was employed as a control for the restriction digestion (9a). *Eco*R1 restriction enzyme digestion of the plasmid DNA from different clones derived from the same ligation yielded different sized inserts (Fig 9a and 9b). This may have resulted from mixing of the DNA bands during DNA gel extraction (section 3.3.3). Four clones from TB5151 (with approximate insert sizes: 420bp, 420bp, 420bp and 570bp respectively) and seven clones from TB4850D (with approximate insert sizes 500bp, 640bp, 680bp, 930bp, 630bp, 250bp and 300bp respectively) were sent for sequencing. The 220bp cloned PCR products from TB4850D (Results no shown) and that of the L5-*M.smegmatis* mc^2155 lysogen control (Fig 9a,) could not be observed from the agarose gels following *Eco*R1 digestion and were not sent for sequencing. However the sequence of the PCR product L5-*M.smegmatis* mc^2155 lysogen was already determined to be of the L5 GP71 origin (section 3.4.2).

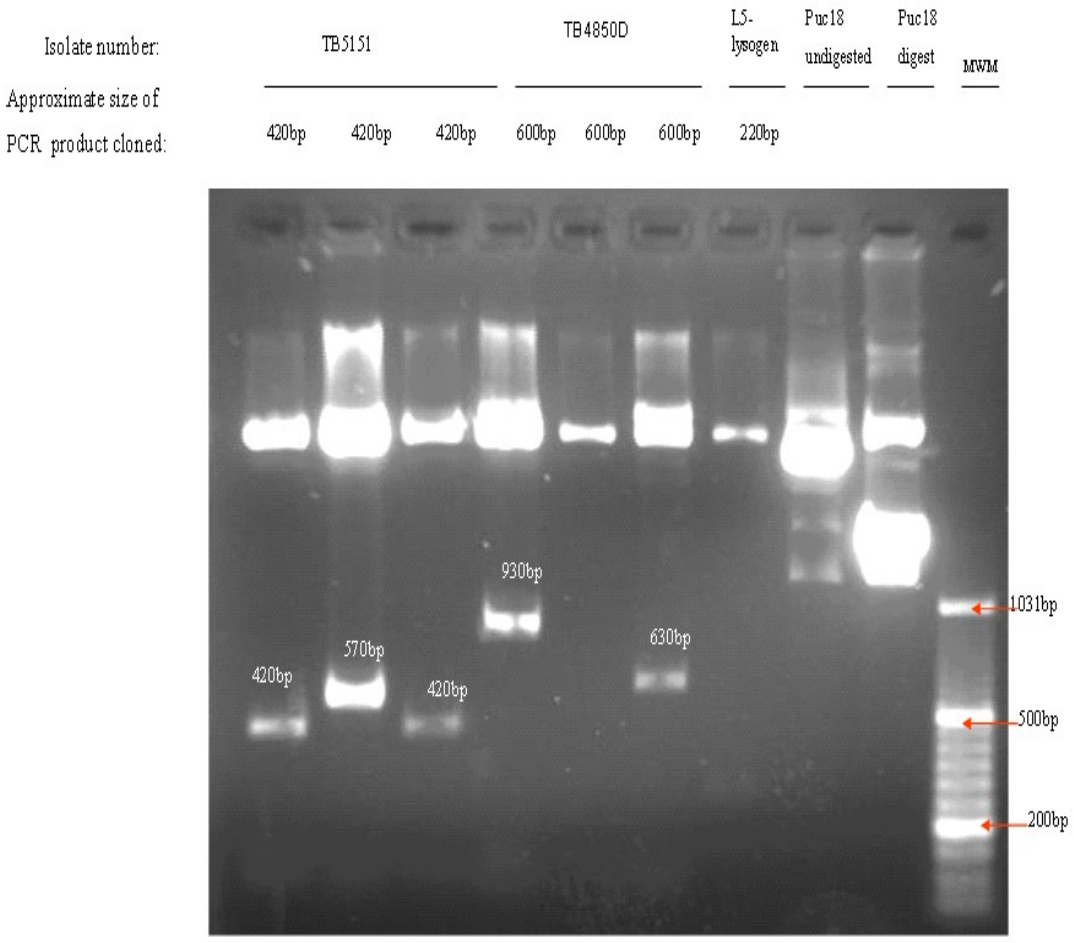

Fig 9a: Restriction products of the plasmid DNA cloned with the PCR products from the guessmer PCR, dgested with *Eco*R1 showing the isolates , their PCR product sizes and the insert sizes. L5 lysogen indicates L5- *M. smegmatis* mc^2155 lysogen. MWM= Molecular Weight Marker (50bp O' range ruler.

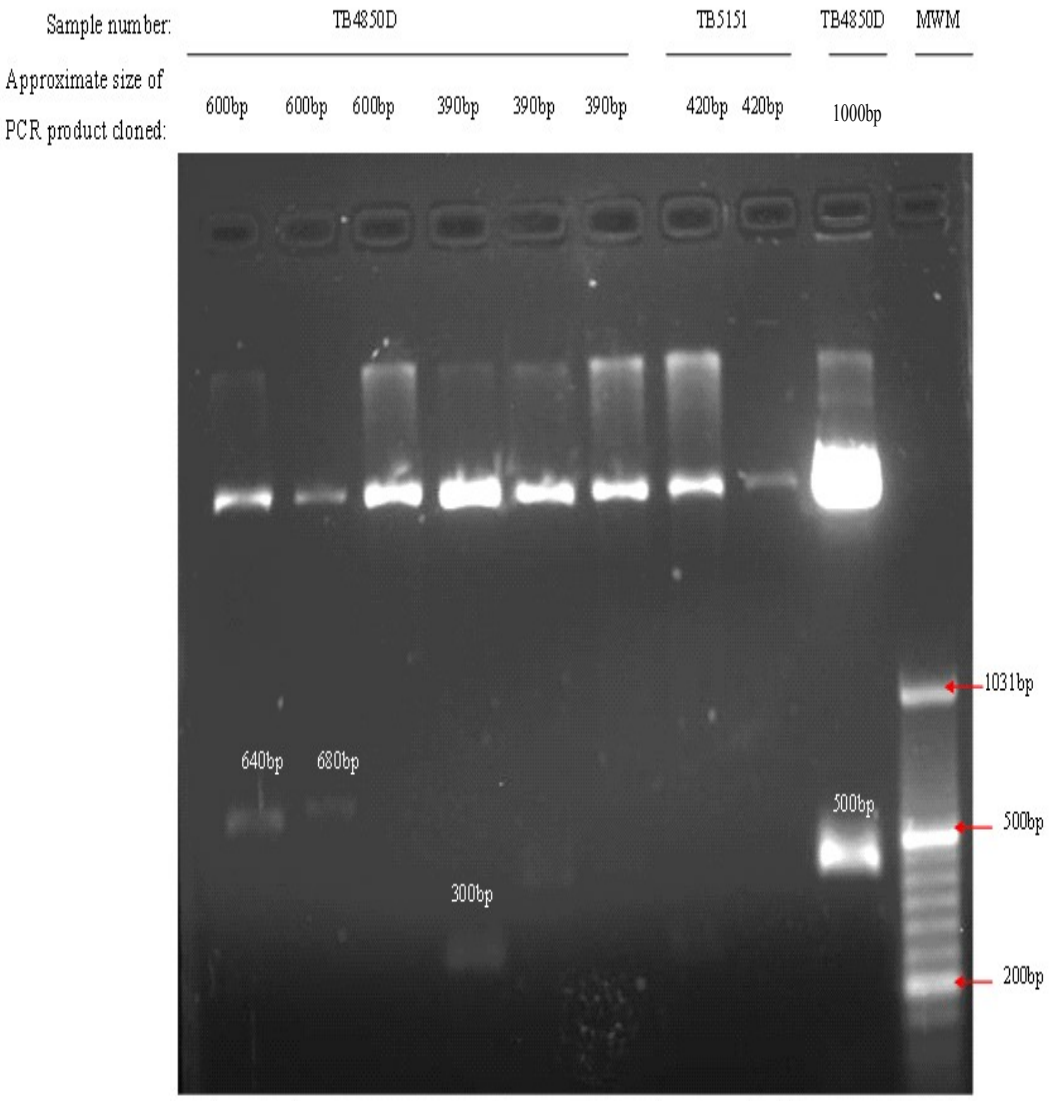

Fig 9b: Restriction products of the plasmid DNA cloned with DNA from guessmer PCR, digested with *Eco*R1 enzymes, showing isolate number, the approximate size of the PCR product and the insert sizes. MWM indicates Molecular Weight Marker (50bp O' range Ruler)

Table 9: *Eco*R1 restriction enzyme digestion of plasmid DNA from different clones, showing insert sizes and the original PCR product sizes.

Isolate number and approximate size of the PCR product cloned	Insert size (bp)	Insert size (bp)	Insert size (bp)	Insert size (bp)	Average insert size (bp)
TB 5151 (420bp*)	420	420	420	570	457.5
TB 4850D (1000bp*)	500	-	-	-	500
TB 4850D (600bp*)	640	680	930	630	720
TB 4850D (390bp*)	-	250	300	-	275
TB 4850D(220bp*)	-	-	-	-	-
L5-*M.smegmatis* mc^2155 lysogen (220bp)*	-	-	-	-	-

- indicates that no DNA insert was observed, *=size of the original PCR product cloned.

3.4.5 Sequence analysis

In order to identify sequences similar to those that we had amplified and sequenced, we searched the NCBI database for similar sequences using the NCBI BLASTX search algorithm, which would allow us to search the translated database. As can be seen in Table 10 and Appendix D, several proteins were identified and all of which were of mycobacterial origin, and had the lowest e-values against *M. bovis* proteins, confirming that our cloned PCR products were of *Mycobacterium bovis* origin. These proteins include probable DNA helicases (Mb0884), transcriptional regulator (Mb1160c), probable IS1533-derived transposases (Mb2968); a hypothetical protein (Mb1618c); a CTP synthetase (Mb1725), and a spermidine synthetase (Mb2632) (BLASTX results: Appendix D). Interestingly the majority of these proteins, i.e. DNA helicases (Mb0884), transcriptional regulator (Mb1160c), probable IS1533-derived transposases (Mb2968) are DNA binding of the helicase superfamily II, of the xenobiotic responsase element family of the transcriptional regulators, and of the intergrases respectively (www.ncbi.nlm.nih.gov/entrez). As can be seen in Appendix D these proteins showed some identical sequences at amino acid level to the region between the sequences

RLRDHGEY and DLLIRVNE and interestingly as can be seen in Appendix D, some of these sequences were identical to the primer sequences used. The CTP synthetase protein showed a 75% and 50% identity to the DLLIRVNE, and RLRDHGEY guessmer sequences respectively. The transcriptional regulator and the DNA helicase proteins revealed 50% and 37.5% identity to the DLLIRVNE sequence respectively. The hypothetical protein sequence (Mb1618c) showed a 37.5% and 25% identity to RLRDHGEY and DLLIRVNE respectively. No sequence similarities to the primers were observed on the spermidine synthetase ((Mb2632), and the IS1553 element.

With the observation that the shared sequence motif in most of these proteins and the repressor-like proteins of the temperate mycobacteriophages is DLLIRVNE, we also did a Sanger Institute BLASTX search using the DLLIRVNE sequence against the *M. bovis* genome, in order to investigate the conservation of this sequence in the *M. bovis* genome (www.sanger.ac.uk). Interestingly this sequence appeared to be also conserved (showing 87.5% conservation) in the glutamine- transport ATP binding abc transporter. A Prosite database search to see if this sequence was identified previously was done using the sequence DLLIRVNE as a search tool, and no hit was found indicating that a possible novel DNA binding motif has been identified (http://www.expasy.ch/prosite/).

Table 10: Homolog search results revealing the predicted homolog proteins and their location on the *M. bovis* genome

Isolate	Query size (bp)	Highest BLAST hit	Other mycobacteria hit	Other genus hit	Genomic location	Insert size (bp)
TB4850D (1000bp*)	634	Probable DNA helicase [*Mycobacterium.bovis* AF2122/97] [2e-69][122-538bp**]	Probable DNA helicase ercc[3][*Mycobacterium tuberculosis* H37Rv] [2e-69]	Putative NDA helicase [norcadia farcinica ifm 10152[2e-57]	959285..960904***	~500
TB4850D (600bp*)	762	Ctp synthetase [*Mycobacterium bovis* AF2122/97][2e-101][115-672bp**]	Ctp synthetase [*Mycoabcterium tuberculosis* CDC1551 [4e-101]	Ctp synthetase [rhodococcus sp. Rha1] [2e-86]	1909166..1910926***	~930
TB5151 (420bp*)	679	Probable transcriptional regulator protein [*Mycobacterium bovis* AF2122/97] [6e-80]116-571**]	Probable transcriptional regulator protein [*Mycobacterium tuberculosis* H37Rv][6e-80]		12544447..1255904***	~420
TB5151 (420bp*)	689	Hypothetical protein Mb1618c [*Mycobacterium bovis* AF2122/97] [2e-77][207-376]	Hypothetical protein *Mycobacterium tuberculosis* CDC1551][2e-77]		1779105..1777768***	~420
TB4850D (390bp*)	327	Spermidine synthetase [*Mycobacterium bovis* AF2122/97][2e-9][123-233**]		Hypothetical protein 3 [micropilis demolitor braccovirus][4e-10] [2-112]	2896169..2897413***	~250
TB4850D (390bp**)	561	IS1533 Transposase [*Mycobacterium bovis* AF2122/97][3e-17][187-312]	IS1533 transposase [*Mycobacterium tuberculosis* H37Rv]		3245037..3246725***	~390

*** indicates the original PCR product size, ** indicates the location of the query amino acid sequences within the BLAST hit, ***=location of the proteins in the *M. bovis genome***

3.5 DISCUSSION

Guessmer PCR based genomic screening for *M. bovis* prophages using primers based on the conserved sequences of repressor proteins of eight sequenced mycobacteriophages were shown to amplify uniquely L5 repressor DNA in a L5 lysogen, yielded multiple bands which occurred in several (30% - of the ones that had good quality hsp65+ DNA) of the screened *M. bovis* samples. Sequence analysis of some of these bands revealed DNA encoding homolog proteins to different *M. bovis* proteins, some of which had affinity to DNA binding proteins.

One of the proteins identified in isolates (TB4850D, and TB5151) was a putative transposase of the insertion sequence family IS1533 (Mb 2968); a putative transcriptional regulators (Mb1160); a probable DNA helicase (Mb0884); a spermidine synthetase, a CTP synthatase (Mb1725) as well as a hypothetical protein (Mb1618c) (Appendix D). An overriding feature of several of the identified proteins is that they are implicated in DNA binding (transcriptional regulator, IS1553 element, and the DNA helicase). As can be seen in appendix D, the shared sequence motif is the DLLIRVNE sequence, which is also conserved in the repressor-like proteins of mycobacteriophages Bxb1, Bxz2, L1, U2 Bethlehem, Che12 and Ms6. DLLIRVNE is also conserved in the phage D29, a lytic phage that shares over 75% of its amino acid sequences with the L5 phage (Pedulla *et al.,* 2003). Interestingly 75%, 50%, 37.5%, and 25% of DLLIRVNE is conserved in the following putative proteins respectively: CTP synthatase (Fig F, Appendix D), the transcriptional regulator (Fig B, Appendix D), the DNA helicase (Fig D, Appendix D) and the hypothetical protein (Mb1618c) (Fig K, Appendix D) of the sequences *M. bovis* AF2122/97 strain and we predicted that these are the regions where our DLLIRVNE guessmer primer bound. In addition, the BLASTX search for conservation of DLLIRVNE in *M. bovis*, using the conserved DLLIRVNE as a query against *M. bovis* AF2122/97 revealed the availability of the 87.5% of DLLIRVNE in probable glutamine-transport ATP binding abc transporter, which is also DNA binding (www.sanger.ac.uk). The function of the transcriptional regulator protein (Mb1160c) is not known, but this protein is perceived to be a specific prokaryotic DNA binding protein belonging to the

xenobiotic responsase element family of the transcriptional regulators (www.sanger.ac.uk;www.ncbi.nlm.nih.gov). This protein is also equivalent to Rv1129 of *M. tuberculosis.* The conservation of DLLIRVNE in DNA binding proteins of *M. bovis* and mycobacteriophages repressors may suggest that this sequence plays a very important role in the DNA binding process. This sequence has not been recognized before as a DNA binding motif in temperate mycobacteriophages. DLLIRVNE is located at the C terminal domain of the mycobacteriophage repressor proteins and the C-terminal domain of the temperate mycobacteriophage repressors such as Che12 and L5 is reported to be responsible for dimerisation of the repressor monomers (Gomathi *et al.,* 2007). Only the helix turn helix motif located at the N- terminal has been reported as a DNA binding motif in phages L5 (Donelly-Wu *et al.,* 1993; Gomathi *et al.,* 2007) Che12 (Gomathi *et al.,* 2007), However it is possible that we might have identified a previously unrecognized DNA binding region.. Another noteworthy observation is its high conservation in the CTP synthetase, which functions for CTP synthesis; hence we also think that this protein might be a DNA binding protein.

We could not conclude that this sequence (DLLIRVNE) is of prophage origin, since it is only a small sequence and the NCBI data base does not contain prophage sequences.

3.6 CONCLUSION

In this chapter, our aim was to screen for mycobacteriophage related sequences, in *M. bovis* isolates using conserved sequences from repressor proteins of different mycobacteriophages. We were aware that the use of these sequences from repressor proteins could be limiting as these phages with almost identical repressors seem to have a common ancestry. However since all sequenced temperate phages known to us belong to this group of 8/30 mycobacteriophage population, that seem to have identical repressor sequences, with 2 blocks of 8 amino acids that is conserved, we reasoned that we could use these sequences to look for their conservation in *M. bovis* prophages. We decided to use repressor proteins as they are the hallmark of temperate phages instead of integrases, which are enormous in many bacterial genomes.

Guessmers were designed from the two conserved sequences and *M. bovis* isolates were screened for prophages using the designed guessmer primers. The BLAST results of the sequences from the PCR products from this screen revealed different proteins of mycobacterium origin, in particular *M. bovis*. Another noteworthy observation was that some of these proteins were DNA binding. We also noted the conservation of the DLLIRVNE sequence in these proteins which is also conserved in the mycobacteriophages repressor-like sequences, and hence we came to a conclusion that this sequence might play a very important role in DNA binding. Hence we also think that the CTP synthetase of *M. bovis* in which this sequence is also very highly conserved might have DNA binding affinity. We however could not conclude that this sequence (DLLIRVNE) is of prophage origin, since there are no prophage sequences on the NCBI database, and an in depth analysis of the whole *M. bovis* genome would be necessary to confirm the origin of this sequence. Our conclusion therefore is that prophage sequences were not identified in the *M. bovis* isolates, but a novel possible DNA binding motif present in a number of proteins, indicating that they all bind DNA as part of their function, has been identified.

CHAPTER FOUR: CONCLUDING REMARKS

The long term aim of this research was to determine the contribution of phages to the virulence and pathogenicity of pathogenic *M. bovis*. The short term aim, which was the aim of this study, was to isolate inducible or cryptic phages from *M. bovis* isolates and characterize them. In this chapter we present the summary of the results and concluding remarks.

Since the low frequency of spontaneous phage induction from *M. bovis* as shown by Tageldin *et al.,* (1981), and no further reports of phage isolation from this bacillus, it was necessary for us to optimize methods for phage isolation, using a well-known phage system. The study of the effect of co-cultivation of a lysogenic *M. smegmatis* mc^2155 and its wild type strain was motivated by the fact that most studies have shown that spontaneous phage induction is very rare, occurring at a rate of 10^2-10^5 in a 10^9 bacterial population, and the situation was proven different in *Salmonella* co-cultures. The result we obtained with *M. smegmatis* co-cultures showed no significant difference in the rate of phage release between the lysogen co-culture with wild type, and the lysogen control cultivated clonally. We reasoned that this might be due to L5 forming stable lysogens with *M. smegmatis.*

The detection of phage particles from solid lysogenic *M. smegmatis* mc^2155 culture by epiflourescence microscopy was motivated by the fact that some studies have shown that plaque assay methods are biased, selecting only phages that can infect that sensitive strain, leading to the detection of only a tiny fraction of phages, compared to non-selective microscopic methods. Epiflourescence microscopy is this study was shown to be more sensitive and feasible for phage detection in solid cultures requiring a titer of at least 10^2 pfu/ml to detect twice the number of individual phage particles detected by plaque assay.

Screening for spontaneously induced phages from *M. bovis* isolates by plaque assay using 5 different bacterial indicator strains was not achieved. In addition screening for

spontaneously induced phages from *M. bovis* isolates using a non-selective epiflourescence microscopy, only revealed a viral like spot in one out of sixteen isolates. However this result alone, without any supporting evidence from Transmission electron microscopy and phage DNA isolation could not be used to conclude the presence of a spontaneously induced phage in that isolate, since only one spot thought to be an artifact was observed. Our conclusion therefore, based on plaque assay results and epiflourescence microscopy, is that spontaneous phage induction in these isolates did not occur.

The genomics approach to screen for the presence of cryptic phages using guessmers designed from conserved regions in the mycobacteriophage repressor-like proteins was motivated by the fact that, repressors are a hallmark of temperate phages. We came to the realization that these phages sharing repressor-like sequences might actually be related or come from a common ancestor. We also noted that characterized temperate mycobacteriophages fall in this group of eight phages, which also made us to think that may be all known temperate mycobacteriophages have identical repressors. Amplification of the *M. bovis* DNA by the guesmers, followed by cloning and sequencing of the PCR products was done. The obtained sequences were homologs of different proteins in the sequenced *M. bovis* genome. Analysis of these sequences revealed that they were DNA binding proteins, with sequences like DLLIRVNE or part of, which was shared by the L5 and the other related mycobacteriophages. The function of this sequence was not obvious. This sequence is highly conserved in temperate mycobacteriophages and available in the obtained DNA binding protein sequences including and a probable glutamine- transport ATP binding abc transporter protein, and a CTP synthatase of the sequenced *M. bovis* genome, hence we concluded that this sequence might play an important role in DNA binding. In order to conclude that these obtained sequences in the *M. bovis* isolates in particular DLLIRVNE is of prophage origin, a more in depth analysis of the whole genome is needed.

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APPENDIX A

Source of chemicals and suppliers

7H9 Middlebrook Broth	Cat No 271310	Difco
7H10 Middlebrook Agar	Cat No 262710	Difco
Ampicilin	Cat No 171254	CalBiochem
BioTaq™ DNA Polymerase (5u/µl)	Cat No BIO-2140	Bioline LTD
DNA Purification kit	Cat No 27-9602-01	GelHealth
Löwenstein Jensen slants		NHLS
Mitomycin C	Cat No M0503	Sigma Aldrich
O' Gene Ruler DNA ladder (50bp)	Cat No SM1133	Fermentas Inc
Oligo Synthesis	Inqaba Biotechnological Industries	
PCR Grade water: Sabax injection Water		Adcock Ingram
PGEMT-Easy Vector System	Cat No A1360	Promega
RNAse A	Cat No 109169	BoehringeMannheim
Restriction enzyme: *EcoR1*	Cat No ER0273	Fermentas

SYBR Green 1	Cat No 1988131	Roche
Sequencing		Inqaba Biotechnical Industries

APPENDIX B

General Media and Buffers

Ampicilin (0.1g/ml)

Add 0.1g of ampicilin powder in 1ml of dH_2O. Filter- sterilize and store at -20ºC.

Indicator plates

Per litre of indicator plate's agar:

19g Middlebrook 7H10 agar

5ml Glycerol

900ml dH_2O

Autoclave, 15min. Allow to cool and add filter sterilized glucose to a final concentration of 0.03%, filter sterilsed Calcium Chloride, to a final concentration of 1mM, autoclaved Magnesium Chloride to a final concentration of 1mM. Add a 100ml of fresh culture of indicator bacterial strain and mix well

Luria Broth and Agar

Per litre of dH_2O

10g Tryptone Powder

5g Yeast Extract

5g NaCl

For agar, 10g of Bacteriological agar. Autoclave 15-20 minutes.

Middlebrook 7H9 Broth

Per litre of dH_2O

4.7g Middlebrook 7H9 powder

4ml Gycerol

Autoclave, 15 minutes. Allow to cool, and add filter sterilized glucose to a final concentration of 0.03%.

Middlebrook 7H10 agar

Per litre of dH_2O

19g Middlebrook 7H10 powder

5ml Glycerol

Autoclave,15 minutes. Allow to cool and add filter sterilized glucose to a final concentration of 0.03%.

Mounting Solution

Per 900µl solution

445µl Phosphate Buffer Saline (PBS)

445µl Glycerol

10µl of 10%p- phenylenediamine (p-p-d)

Add all the reagents and mix thoroughly. Must always be freshly prepared.

Phage Buffer

Per litre dH_2O

10mM Tris (pH=8)

10mM $MgSO_4$

68mM NaCl

10mM $CaCl_2$

Add about 800ml of dH_2O to a mixture of Tris, $MgS0_4$ and NaCl. Adjust the pH to 7.8-8. Add dH_2O to a final volume of 1000ml and autoclave. Add filter sterilized Calcium Chloride to a final concentration of 10mM. Store at room temperature or 4ºC.

Phosphate Buffer Saline (PBS)

Per litre dH_2O

8g NaCl

0.2g KCl,

1.44g Na_2HPO_4

0.24g KH_2PO_4

Add about 800ml dH2O and mix. Adjust the pH to 7.4. Add dH_2O to a final volume of 1000ml. Autoclave, 15-20 min. Allow to cool. Store at room temperature.

10% p-phenylenediamine (p-p-d)

Add 0.1g of p-p-d powder or granules to 1ml sdH_2O. Heat gently in boiling water until dissolved. Allow to cool and use immediately.

RNAse A (10mg/ml)

Per 1ml

Add 10mg of RNAse A powder in 1ml of 10mM Tris-HCl (pH=8), and 15mM NaCl solution. Heat to 100ºC in a beaker of boiling water for 15min. Store at -20ºC.

Soft Agar for Overlays

Prepare 0.9% Luria or 7H9 or 7H10 agar. Autoclave for 15-20 minutes

Solution 1

50mM glucose
25mM Tris. Cl (pH =8)
10mM EDTA (pH=8)

Autoclave for 15 minutes and store at 4 ºC

Solution II

0.2N NaOH (freshly prepared from a 10N stock)
1% SDS (Autoclaved)
Mix well but do not vortex. This is always freshly prepared

Solution III

For 100ml solution:

5M potassium Acetate (Autoclaved)
Glacial Acetic acid
sdH_2O

Add 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid and 28.5ml dH_2O. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. Store at 4ºC.

10x TAE Buffer

Per Litre of dH2O

48.4g Tris Base

11.42 ml Glacial Acetic acid

20ml of 1.5 M EDTA (pH=8)

Make up to a litre. Autoclave for 20 minutes.

Transformation Buffer

Per 100ml dH20

2.2g CaCl2. $2H_20$

1ml of 1M Tris.HCl (pH=8)

Add sdH_2O to a final concentration of 100ml. Autoclave for 15minutes. Cool and store at 4ºC.

APPENDIX C

pGEM-T Easy Vector Map and sequence reference points

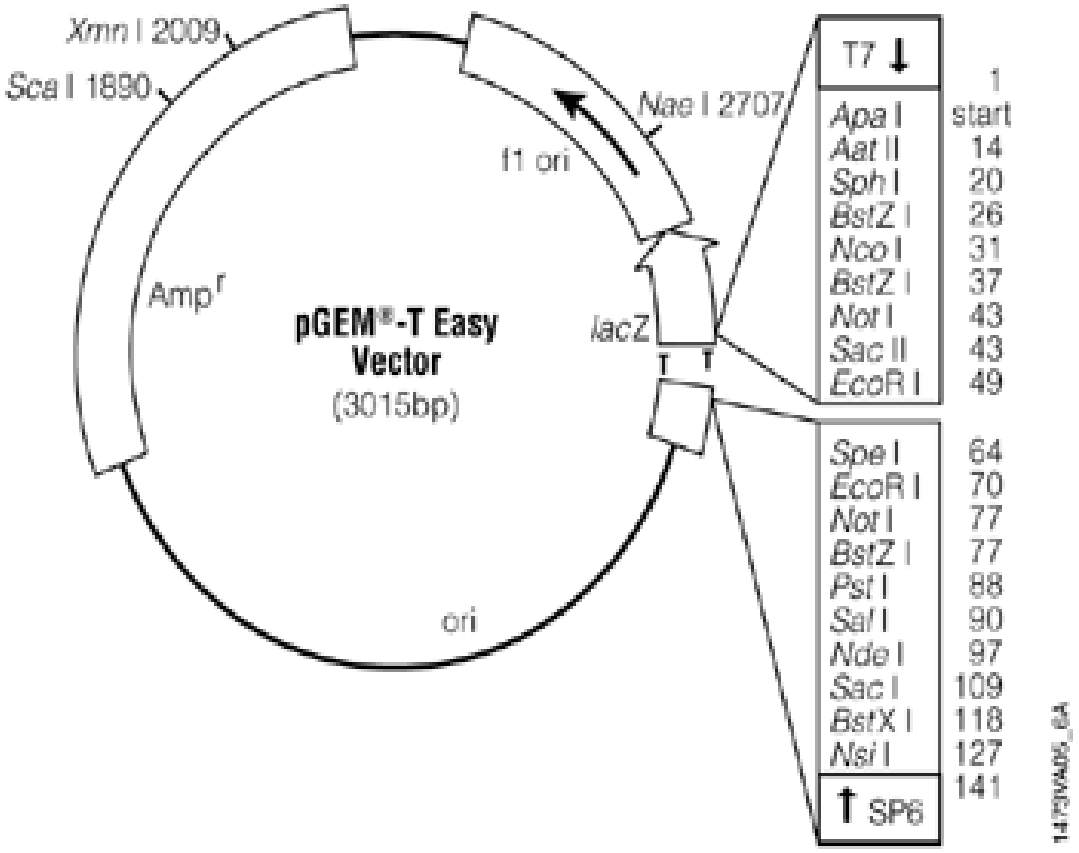

Fig extracted from: www.promega.com.

APPENDIX D

NCBI BLASTX

Query 194 ERMFTESGMRTGGLDIQLAELMRDRFGISVVIDDNLPDTAKRRYHPDTKVLRVAHWLMPG 373
ERMFTESGMRTGGLDIQLAELMRDRFGISVVIDDNLPDTAKRRYHPDTKVLRVAHWLMPG
Sbjct 177 ERMFTESGMRTGGLDIQLAELMRDRFGISVVIDDNLPDTAKRRYHPDTKVLRVAHWLMPG 236

Query 374 QRAFQIATQLALVGQSDLISSIVATDDQLSTEARGVARIGLANYFAGAFLLPYREFHRAA 553
QRAFQIATQLALVGQSDLISSIVATDDQLSTEARGVARIGLANYFAGAFLLPYREFHRAA
Sbjct 237 QRAFQIATQLALVGQSDLISSIVATDDQLSTEARGVARIGLANYFAGAFLLPYREFHRAA 296

Query 554 EQLRYDIDLL 583
EQLRYDIDLL
Sbjct 297 EQLRYDIDLL 306

Fig A: NCBI BLASTX results of a 679bp sequence from isolate TB5151 used as a query revealing homology with a transcriptional regulator protein (Mb1160) of *M, bovis* AF2122/97 . The sequence shaded in red is identical to the guessmer primer sequence DLLIRVNE .

MTRSNVLPVA RTYSRTFSGA RLRRLRQERG LTQVALAKAL DLSTSYVNQL ENDQRPITVP
61 VLLLLTERFD LSAQYFSSDS DARLVADLSD VFTDIGVEHA VSGAQIEEFV ARMPEVGHSL
121 VAVHRRLRAA TEELEGYRSR ATAETELPPA RPMPFEEVRD FFYDRNNYIH DLDMAAERMF
181 TESGMRTGGL DIQLAELMRD RFGISVVIDD NLPDTAKRRY HPDTKVLRVA HWLMPGQRAF
241 QIATQLALVG QSDLISSIVA TDDQLSTEAR GVARIGLANY FAGAFLLPYR EFHRAAEQLR
301 YDIDLLGRRF GVGFETVCHR LSTLQRPRQR GIPFIFVRTD KAGNISKRQS ATAFHFSRVG
361 GSCPLWVVHD AFAQPERIVR QVAQMPDGRS YFWVAKTTAA DGLGYLGPHK NFAVGLGCDL
421 AHAHKLVYST GVVLDDPSTE VPIGAGCKIC NRTSCAQRAF PYLGGRVAVD ENAGSSLPYS
481 STEQSV

Fig B: Amino acid sequence of the Transcriptional regulator (Mb 1160) protein of *M, bovis* AF2122/97, retrieved from the NCBI database (accession number: NP_854816). The sequence shaded in red is the predicted binding site of the guessmer primer (DLLIRVNE).

Query 122 HSDEEVLVIGAYLDQLDELGAELGAPVIQGSTRTSEREALFDAFRRGEVATLVVSKVANF 301
H DE+ LVIGAYLDQLDELGAELGAPVIQGSTRTSEREALFDAFRRGEVATLVVSKVANF
Sbjct 400 HPDEQTLVIGAYLDQLDELGAELGAPVIQGSTRTSEREALFDAFRRGEVATLVVSKVANF 459

Query 302 SIDLPEAAVAVQVSGTFGSRQEEAQRLGRILRPKADGGGAIFYSVVARDSLDAEYAAHRQ 481
SIDLPEAAVAVQVSGTFGSRQEEAQRLGRILRPKADGGGAIFYSVVARDSLDAEYAAHRQ
Sbjct 460 SIDLPEAAVAVQVSGTFGSRQEEAQRLGRILRPKADGGGAIFYSVVARDSLDAEYAAHRQ 519

Query 482 RFLAEQGYGYIIRDADDLL 538
RFLAEQGYGYIIRDADDLL
Sbjct 520 RFLAEQGYGYIIRDADDLL 538

Fig C: BLASTX results of a 634bp sequence from isolate TB4850D used as a query revealing homology with a DNA helicase(Mb0884) protein of *M, bovis* AF2122/97 . The sequence shaded in red is part of the identical sequence used in the primers

MQSDKTVLLE VDHELAGAAR AAIAPFAELE RAPEHVHTYR ITPLALWNAR AAGHDAEQVV
61 DALVSYSRYA VPQPLLVDIV DTMARYGRLQ LVKNPAHGLT LVSLDRAVLE EVLRNKKIAP
121 MLGARIDDDT VVVHPSERGR VKQLLLKIGW PAEDLAGYVD GEAHPISLHQ EGWQLRDYQR
181 LAADSFWAGG SGVVVLPCGA GKTLVGAAAM AKAGATTLIL VTNIVAARQW KRELVARTSL
241 TENEIGEFSG ERKEIRPATI STYQMITRRT KGEYRHLELF DSRDWGLIIY DEVHLLPAPV
301 FRMTADLQSK RRLGLTATLI REDGREGDVF SLIGPKRYDA PWKDIEAQGW IAPAECVEVR
361 VTMTDSERMM YATAEPEERY RICSTVHTKI AVVKSILAKH PDEQTLVIGA YLDQLDELGA
421 ELGAPVIQGS TRTSEREALF DAFRRGEVAT LVVSKVANFS IDLPEAAVAV QVSGTFGSRQ
481 EEAQRLGRIL RPKADGGGAI FYSVVARDSL DAEYAAHRQR FLAEQGYGYI IRDADDLLGP
541 AI

Fig D : Amino acid sequence of DNA helicase (Mb0884) protein of *M, bovis* AF2122/97, retrieved from the NCBI database (accession number NP_854542) . The sequence shaded in red is the predicted binding site of the primer sequence (DLLIRVNE) .

Query 672 GEYLGDTVQVIPHITDEIKRRILAMAQPDADGNRPDVVITEIGGTVGDIESQPFLEAARQ 493
GEYLGDTVQVIPHITDEIKRRILAMAQPDADGNRPDVVITEIGGTVGDIESQPFLEAARQ
Sbjct 112 GEYLGDTVQVIPHITDEIKRRILAMAQPDADGNRPDVVITEIGGTVGDIESQPFLEAARQ 171

Query 492 VRHYLGREDVFFLHVSLVPYLAPSGELKTKPTQHSVAALRSIGITPDALILRCDRDVPEA 313
VRHYLGREDVFFLHVSLVPYLAPSGELKTKPTQHSVAALRSIGITPDALILRCDRDVPEA
Sbjct 172 VRHYLGREDVFFLHVSLVPYLAPSGELKTKPTQHSVAALRSIGITPDALILRCDRDVPEA 231

Query 312 LKNKIALMCDVDIDGVISTPDAPSIYDIPKVLHREELDAFVVRRLNLPFRDVDWTEWDDL 133
LKNKIALMCDVDIDGVISTPDAPSIYDIPKVLHREELDAFVVRRLNLPFRDVDWTEWDDL
Sbjct 232 LKNKIALMCDVDIDGVISTPDAPSIYDIPKVLHREELDAFVVRRLNLPFRDVDWTEWDDL 291

Query 132 LIRVNE 115
L RV+E
Sbjct 292 LRRVHE 297

Fig E: NCBIBLASTX results of a 762bp sequence from isolate TB4850D used as a query revealing homology with CTP Symthetase (Mb 1725) protein of *M, bovis* AF2122/97 . The sequence shaded in red is identical to the primer

MRKHPQTATK HLFVSGGVAS SLGKGLTASS LGQLLTARGL HVTMQKLDPY LNVDPGTMNP
61 FQHGEVFVTE DGAETDLDVG HYERFLDRNL PGSANVTTGQ VYSTVIAKER RGEYLGDTVQ
121 VIPHITDEIK RRILAMAQPD ADGNRPDVVI TEIGGTVGDI ESQPFLEAAR QVRHYLGRED
181 VFFLHVSLVP YLAPSGELKT KPTQHSVAAL RSIGITPDAL ILRCDRDVPE ALKNKIALMC
241 DVDIDGVIST PDAPSIYDIP KVLHREELDA FVVRRLNLPF RDVDWTEWDD LLRRVHEPHE
301 TVRIALVGKY VELSDAYLSV AEALRAGGFK HRAKVEICWV ASDGCETTSG AAAALGDVHG
361 VLIPGGFGIR GIEGKIGAIA YARARGLPVL GLCLGLQCIV IEAARSVGLT NANSAEFDPD
421 TPDPVIATMP DQEEIVAGEA DLGGTMRLGS YPAVLEPDSV VAQAYQTTQV SERHRHRYEV
481 NNAYRDKIAE SGLRFSGTSP DGHLVEFVEY PPDRHPFVVG TQAHPELKSR PTRPHPLFVA
541 FVGAAIDYKA GELLPVEIPE IPEHTPNGSS HRDGVGQPLP EPASRG

Fig F: Amino acid sequence of CTP Symthetase (Mb 1725) protein of *M, bovis* AF2122/97, retrieved from the NCBI database (accession number NP_855378) . The sequence shaded are the predicted binding sites of the guessmer primers.

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Query  187   TYVAGEIGQCDFWFPDVVVPVGYGQVRTATALPVLTMVCGYS  312
             TYVAGEI QCDFWFP + +PVG+GQ RTA  LPVLTMVC YS
Sbjct 112  TYVAGEIAQCDFWFPPIELPVGFGQTRTAKQLPVLTMVCAYS  153

Query 129  SWRVRELRPLYLPPDPASR  185
             S RV ELRP+YLPPDPASR
Sbjct 92   SARVAELRPVYLPPDPASR  110
```

Fig G: NCBI BLASTX results of a 561bp sequence from isolate TB4850D used as a query revealing homology with Insertion sequence element (IS) 1553 Transposase (Mb 2968) protein of *M, bovis* AF2122/97 .

MLTVEDWAEI RRLHRAEGLP IKMIARVLGI SKNTVKSALE SNQQPKYERA PQGSIVDAVE
61 PRIRELLQAY PTMPATVIAE RIGWERSIRV LSARVAELRP VYLPPDPASR TTYVAGEIAQ
121 CDFWFPPIEL PVGFGQTRTA KQLPVLTMVC AYSRWLLAML LPSRCAEDLF AGWWRLIEAL
181 GAVPRVLVWD GEGAIGRWRG GRSELTTECQ AFRGTLAAKV LICRPADPEA KGLIERAHDY
241 LERSFLPGRV FASPADFNAQ LGAWLALVNT RTRRALGCAP TDRIGADRAA MLSLPPVAPA
301 TGWCTSLRLP RDHYVRCDSN DYSVHPGVIG HRVLVRADLE RVHVFCDGEL VADHERIWAV
361 HQTVSDPAHV EAAKVLRRRH FSAASPVVEP QVQVRSLSDY DDALGVDIDG GVA

Fig H : Amino acid sequence of IS 1553 (Mb 2968) protein of *M, bovis* AF2122/97, retrieved from the NCBI database (NP_856613) .

Query 233 E*VVQVELDPAVIELARTTLRDVNAGSLDNPRVLAVI 123

E + VQVELDPAVIELARTTLRDVNAGSLDNPRV VI

Sbjct 327 EQIVQVELDPAVIELARTTLRDVNAGSLDNPRVHVVI 363

Fig I : NCBI BLASTX results of a 327bp sequence from isolate TB4850D used as a query revealing homology with Spermidine synthetase (Mb2632) protein of *M, bovis* AF2122/97 .

MTSTRQAGEA TEASVRWRAV LLAAVAACAA CGLVYELALL TLAASLNGGG IVATSLIVAG
61 YIAALGAGAL LIKPLLAHAA IAFIAVEAVL GIIGGLSAAA LYAAFAFLDE LDGSTLVLAV
121 GTALIGGLVG AEVPLLMTLL QRGRVAGAAD AGRTLANLNA ADYLGALVGG LAWPFLLLPQ
181 LGMIRGAAVT GIVNLAAAGV VSIFLLRHVV SGRQLVTALC ALAAALGLIA TLLVHSHDIE
241 TTGRQQLYAD PIIAYRHSAY QEIVVTRRGD DLRLYLDGGL QFCTRDEYRY TESLVYPAVS
301 DGARSVLVLG GGDGLAAREL LRQPGIEQIV QVELDPAVIE LARTTLRDVN AGSLDNPRVH
361 VVIDDAMSWL RGAAVPPAGF DAVIVDLRDP DTPVLGRLYS TEFYALAARA LAPGGLMVVQ
421 AGSPYSTPTA FWRIISTIRS AGYAVTPYHV HVPTFGDWGF ALARLTDIAP TPAVPSTAPA
481 LRFLDQQVLE AATVFSGDIR PRTLDPSTLD NPHIVEDMRH GWD

Fig J: Amino acid sequence of Spermidine synthase (Mb 2632) protein of *M, bovis* AF2122/97 tretrieved from the NCBI database (accession number: NP-856278) .

Query 645GGRGNSISLSDHGEYAPDLDIVGAVLGSPVGDLGHTFRRLNGTllaglpalvvaaLQHSY 466

GG ++ + GEYAPDLDIVGAVLGSPVGDLGHTFRRLNGTLLAGLPALVVAALQHSY

Sbjct 207 GGLASAWAAEACGEYAPDLDIVGAVLGSPVGDLGHTFRRLNGTLLAGLPALVVAALQHSY 266

Query 465 PGLARVIKEHANDEGRQLLEQLTEMTTVDAVIRMAGRDMGDFLDEPLEDILSTPEVSHVF 286

PGLARVIKEHANDEGRQLLEQLTEMTTVDAVIRMAGRDMGDFLDEPLEDILSTPEVSHVF

Sbjct 267 PGLARVIKEHANDEGRQLLEQLTEMTTVDAVIRMAGRDMGDFLDEPLEDILSTPEVSHVF 326

Query 285 GDTKLGSAVPTPPVLIVQAVHDYLIDVSDIDALADSYTAGGANVTYHRDL 136

GDTKLGSAVPTPPVLIVQAVHDYLIDVSDIDALADSYTAGGANVTYHRDL

Sbjct 327 GDTKLGSAVPTPPVLIVQAVHDYLIDVSDIDALADSYTAGGANVTYHRDL 376

Fig K : NCBI BLASTX results of a 689bp sequence from isolate TB5151 used as a query revealing homology with a hypothetical protein (Mb1618c) of *M, bovis* AF2122/97 . The sequence shaded in red is identical to the primer sequences used

MVEPGNLAGA TGAEWIGRPP HEELQRKVRP LLPSDDPFYF PPAGYQHAVP GTVLRSRDVE
61 LAFMGLIPQP VTATQLLYRT TNMYGNPEAT VTTVIVPAEL APGQTCPLLS YQCAIDAMSS
121 RCFPSYALRR RAKALGSLTQ MELLMISAAL AEGWAVSVPD HEGPKGLWGS PYEPGYRVLD
181 GIRAALNSER VGLSPATPIG LWGYSGGGLA SAWAAEACGE YAPDLDIVGA VLGSPVGDLG
241 HTFRRLNGTL LAGLPALVVA ALQHSYPGLA RVIKEHANDE GRQLLEQLTE MTTVDAVIRM
301 AGRDMGDFLD EPLEDILSTP EVSHVFGDTK LGSAVPTPPV LIVQAVHDYL IDVSDIDALA
361 DSYTAGGANV TYHRDLFSEH VSLHPLSAPM TLRWLTDRFA GKPLTDHRVR TTWPTIFNPM
421 TYAGMARLAV IAAKVITGRK LSRRPL

Fig L: Amino acid sequence of hypothetical (Mb 1618c) of *M, bovis* AF2122/97 retrieved from NCBI database, (accession number NP-855271) . The sequences shaded in red is the predicted binding site of the primers .