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Isolation and identification of environmental mycobacteria and associated temperate phages

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CHAPTER ONE - INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Mycobacteria are a group of bacteria that are responsible for many infections in humans and animals world wide. This group can be classified into three main groups. Firstly, the major groups of these organisms that pose the biggest threat are the *M. tuberculosis* complex which can cause tuberculosis-like disease. These include *M. bovis*, *M. africanum* and *M. microti*. Members of the *M. tuberculosis* complex are not found in the environment. The second group is *M. leprae* which is the causative agent of leprosy. The last group constitutes the nontuberculous mycobacteria (NTM), which are all the environmental mycobacteria that may cause various disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease as described by O'Brien *et al*. (1987).

Examples of NTM that cause animal and human disease are other members of the MAC, *M. kansasii, M. fortuitum, M. chelonae, M xenopi*, and *M. abscessus*. The NTM are an increasing health risk which is explained by the increase in the number of susceptible or immunocompromized individuals such as those suffering from acquired immunodeficiency syndrome (AIDS) (Wallace *et al*., 1990; Katoch, 2004). Mycobacteria are normal inhabitants of a wide variety of environments, including soil, water, aerosols, protozoan, animals and humans. The mycobacteria described above can be further divided according to their growth rate: the fast-growers, and the slow-growers. Fast growers predominantly include the NTM while *M. tuberculosis* complex and *M. leprae* are classed as slow-growers.

In addition to the potential health risk associated with NTM, there is also the added role these species may play in the evolution of the group as a whole, particularly their pathogenic counterparts through genetic exchange. It has been suggested that mycobacteria-associated phages (mycobacteriophages) may make an important contribution to the evolution of pathogenic mycobacteria where they can facilitate the horizontal transfer of patho-adaptive alleles between different mycobacteria species and strains (Pedulla *et al*., 2003).

Due to the importance of the mycobacteria as pathogens in both humans and animals, it is necessary to understand the interaction between mycobacteria and associated phages in their environment where very little is known. To this end, we initiated a project to isolate environmental mycobacteria and assess the prevalence of associated phages.

1.2. Literature review

1.2.1. Environmental mycobacteria

Tuberculosis of humans and animals remains one of the most important diseases associated with serious health, economic and social consequences in developed and developing countries. Many publications have been written about the mycobacterial agents of the infection in humans and animals. Less attention has however been devoted to the study of the potentially pathogenic environmental mycobacteria.

The environment is considered the most likely source of NTM infections since members of environmental mycobacteria, that are increasingly implicated in a variety of human and animal infections, are isolated from natural and municipal water; drinking water biofilms; soil and dust; and aerosols (Primm *et al.*, 2004). Table 1 shows a list of environmental mycobacteria species and different locations where they have been isolated from.

1.2.1.1. Soil-associated NTM

A wide variety of soils, sediments and peats have been reported to contain diverse environmental mycobacteria. For example soils including those on river banks, in swamps of the coastal south-eastern United States and forest soils in Finland have all yielded high numbers of *M. avium* due to its innate tolerance to low pH, and low oxygen (Kirschner *et al.,* 1992). Other mycobacteria such as *M. kansasii* and the rapidly growing species *M. smegmatis* and *M. fortuitum* have been shown to be normal inhabitants of soil (Jones and Jenkins, 1965). The impact of human intervention seems to have little to do with the prevalence of NTM as Katila *et al*. (1995) showed that soils from unpolluted areas of Finland contained high levels of

NTM species such as MAC, *M. gordonae*, *M. malmoense*, *M. simiae*, and *M. marinum*.

Another potential source of mycobacteria is potting soils. Soil samples collected from potted plants were shown to yield mycobacteria frequently (89%), including in some cases the well characterised pathogenic NTM's *M. avium* (27%) and *M. intracellulare* (28%). Moreover, freshly opened bags of commercial potting soil also contain *M. avium* and *M. intracellulare* (Yajko *et al.,* 1995).

1.2.1.2. Water and surface-associated NTM

Even though the NTM are normal inhabitants of a wide variety of environments, including soil, aerosols, protozoans, animals and humans (Eaton *et al*., 1995; Laukkanen *et al.,* 2000), water is also a major source of this organism and importantly is the primary source of MAC infection in humans (Goslee and Wolinsky, 1976). Restriction fragment length polymorphism analysis of *M. avium* isolates from AIDS patients were identical to those of isolates recovered from the patients' drinking water (Von Reyn *et al*., 1994). Their prevalence at high levels in the water supply has certainly contributed to the high infection rates seen there (Ristola *et al*., 1999). *Mycobacterium mucogenicum*, *M. kansasii*, *M. gordonae* and *M. flavescens* are among the many other species of environmental mycobacteria isolated from public potable water (Kubalek and Komenda, 1995; Covert *et al.,* 1999). The prevalence of many species of environmental mycobacteria in municipal drinking water supplies (Falkinham *et al.,* 2001) can be explained by their high innate chlorine and biocide resistance (Taylor *et al.,* 2000; Le Dantec *et al*., 2002). The swimming pool provides a suitable habitat for the survival and reproduction of some mycobacteria because of this innate chlorine resistance. The most frequent species found in swimming pools are *M. gordonae, M. chelonae* and *M. fortuitum* (Leoni *et al*., 1999).

In addition to chlorine resistance, the ability of mycobacteria such as *M. fortuitum*, *M. chelonae* and *M. avium* to associate with surfaces in the form of biofilms (Hall-Stoodley and Lappin-Scott, 1998; September *et al*., 2004) has facilitated their adaptation to water, despite their relatively slow growth. Biofilms are formed in distribution system pipelines when microbial cells attach to pipe surfaces and multiply

to form a film or slime layer on the pipe. Biofilms are complex and dynamic microenvironments, encompassing processes such as metabolism, growth and product formation. Biofilms in drinking water pipes can be responsible for a reduction in water quality through increased bacterial levels. The occurrence and growth of environmental mycobacteria in biofilms in drinking water distribution systems may lead to dissemination into the bulk water, constituting a risk to consumers (September *et al*., 2004). Hall-Stoodley and Lappin-Scott (1998) reported that biofilm formation may be a contributing factor to the transmission of *M. fortuitum* and *M. chelonea*. These organisms may be present at high densities in biofilms on the insides of pipes and taps (Le Dantec *et al*., 2002). Biofilms in drinking water distribution systems appear to be rich sources of mycobacteria (Schulze-Röbbecke and Fischeder, 1989), and might have a role in the survival of virulent strains in the environment (George *et al.,* 2003). Environmental mycobacteria may also be adapted to the low nutrient levels often prevalent in water, particularly of municipal origin, since they have relatively high starvation survival traits (Smeulders *et al*., 1999).

The environmental tolerance has certainly contributed to the prevalence of mycobacteria in the water environment, e.g. *M. intracellulare* persisted with only one log loss of viability after 1.4 years in deionized sterile water (Archuleta *et al*., 2002). Furthermore, the tolerance of mycobacteria to temperature extremes (Schulze-Röbbecke and Buchholtz, 1992), results in their contamination of hot tap water and spas with members of the MAC, *M. xenopi*, *M. phlei*, and *M. chelonae* being the most thermo-resistant species. A variety of physiological characteristics of *M. avium* and *M. intracellulare* contribute to their high prevalence in these environments. A *M. avium* and *M. intracellulare* have an acidic pH optimum for growth between 4.5 and 5.5 (George and Falkinham, 1986) and their growth is stimulated by humic and fulvic acid, the major organics in peat and brown water swamps (Kirschner *et al*., 1999).

Large numbers of *M. avium* and *M. intracellulare* have also been recovered from waters and soils with low oxygen levels (Brooks *et al.,* 1984; Kirschner *et al.,* 1992), and representatives of these species can grow under microaerobic conditions (Primm *et al*., 2004). Thus, the mycobacteria are ideally suited for life in those environments. Furthermore, adaptations to acid and micro-aerobic conditions have possibly aided in the virulence of the mycobacteria (Primm *et al.,* 2004).

Table 1. Environmental sites where mycobacteria have been isolated

Species	**Location (Soil and Water)**	**Reference**:
M. gordonae, MAC	City aquarium, drinking water	Goslee and Wolinsky, 1976
MAC	Residential water sources (i.e. toilet, tap, shower)	Von Reyn *et al*., 2002
MAC	Numerous municipal potable water sources	Aronsom *et al*., 1999
M. fortuitum	Sewage treatment plant	Berekaa and Steinbuchel, 2000
M. scrofulaceum, MAC, *M. kansasii*, *M. fortuitum*, & others	Surface water (Rio Grande)	Bland *et al*., 2005
M. fortuitum	Soil (in Malawi)	Brandt *et al*., 2002
MAC	Soil	Brooks *et al.,* 1984
M. scrofulaceum, MAC, *M. szulgai*, *M. fortuitum*, *M. gordonae*, & *M. simiae*	Hospital tap water (in Taiwan)	Chang *et al*., 2002
M. flavescens, *M. austroafricanum*, *M. chlorophenolicum*, & unknown	Petroleum-contaminated soil	Cheung and Kindle, 2001
M. mucogenicum, *M. kansasii*, *M. gordonae*, MAC, *M. fortuitum*, & others	Public drinking and potable water sources, ice machines, water treatment plant	Covert *et al*., 1999
MAC	Hot tubs	Embil *et al*., 1997
M. terrae, MAC, & *M. scrofulaceum*	Water-damaged buildings in Finland	Huttunen *et al*., 2000; Jussila *et al*., 2002
MAC, *M. gordonae*, *M. fortuitum*, & *M. kansasii*	Public swimming pools and whirlpools	Havelaar *et al*., 1985
M. xenopi & *M. botniense*	Natural surface water streams in Finland	Torkko *et al*., 2000
M. marinum, *M. chelonae*, *M. gordonae*, *M. fortuitum*, & others	Public swimming pools in Italy	Leoni *et al*., 1999
M. ulcerans	Natural waters, soil, insects, wild animals, fish	Portaels *et al*., 2001
M. manitobense	Soil sample from Shaganji area of Agra in India	Parashar *et al*., 2007
M. vanbaalenii, M. mageritense, M. frederiksbergense, M. austroafricanum & M. chubuense	Soils at an illegal dumping site and landfills in Japan	Wang *et al*., 2006
M. ulcerans 00-1441	Aquatic hemiptera in Benin	Portaels *et al*., 2008

Table adapted from Primm *et al*. (2004)

1.2.2. Non-Tuberculosis Mycobacterial (NTM) Diseases

Diverse diseases are associated with NTM. The most common associated with pulmonary disease are MAC, *M. fortuitum*, *M. gordonae, M. marinum, M. scrofulaceum, M. terrae, M. ulcerans, M. xenopi, M. chelonae, M. immunogenum, M. abscessus, M. kansasii, M. szulgai, M. simiae* and *M. palstre* (O'Brien *et al*., 1987). These microbes are found in many places in the environment: tap water, fresh and

ocean water, milk, bird droppings, soil, and house dust. The manner in which these bacteria are transmitted is not completely understood. There is no evidence that they are transmitted from person to person.

The NTM are a rare cause of lung disease in otherwise healthy humans but a frequent cause of infection among those whose resistance has been lowered by other disorders and diseases such as HIV. For example, MAC infection is a common complication of HIV infection and has been implicated in a range of human diseases including pulmonary and consequent disseminated diseases in immunocompromised patients (Embil *et al*., 1997; Brown-Elliott and Wallace, 2002). Members of the MAC may cause progressive lung disease leading to respiratory failure and even death in previously healthy patients, usually elderly women without a history of lung disease or immunodeficiency (Prince *et al*., 1989). However, as is shown in Table 2, the type of disease may not only depend on the causative agent, but also the infected host, since members of the MAC and *M. scrofulaceum* have been reported cause of inflammation of the lymph nodes in human (Wallace *et al*., 1990; Sachdeva *et al*., 2002). *Mycobacterium fortuitum* and *M. chelonae* cause skin and wound infections as well as abscesses after trauma or surgical procedures. Other NTM such as *M. marinum* may cause a nodular inflammation of the arms and legs. This infection is called "swimming pool granuloma" because it is often associated with swimming pools, fish tanks, and other bodies of water (Prinner, 1935; Collins *et al*., 1985). While *M. ulcerans* infection causes chronic skin ulcerations, usually on an arm or leg in human.

Table 2. Mycobacterial diseases and source of contamination

Organism	Pulmonary Disease and other	Source	Reference:
MAC	Pulmonary disease	Hot tub	Embil *et al.,* 1997
M. chelonae	Keratitis or corneal ulcers	Contaminated endoscopy	Kressel and Kidd, 2001
M. immunogenum	Hypersensitivity pneumonitis	Metal removal fluids	Shelton *et al*., 1999
M. ulcerans	Ulcerative disease	Irrigation waters	Ross *et al.,* 1997
M. marinum	Skin infections	Swimming pool	Wolinsky, 1979
M. fortuitum	Soft tissue lesions	Soil	Wolinsky, 1979
M. paratuberculosis	Crohn's disease		McFadden *et al*., 1987
M. kansasii	Unlike tuberculosis, cavitary lung disease		Sachdeva *et al*., 2002
M. scrofulaceum	Pulmonary disease		Sanders *et al*., 1995
M. xenopi	Pulmonary disease	Hot water	Wallace *et al*., 1990

1.2.3. The associated mycobacteriophages

1.2.3.1. Bacteriophages

Bacteriophages (phages) are intracellur parasites that infect bacteria. Bacteriophages contain nucleic acids, an essential component of living organisms, as single or double stranded forms of RNA or DNA. The genome is surrounded by a protein coat, which may be covered by a lipid layer. They have no self-replicating mechanism and so rely on parasiting living host cells to replicate. Consequently they lack the ability of all living organisms in possessing the ability to reproduce and instead must make their host reproduce more phages. Phage do not all replicate in the same manner as some do not transcribe their DNA, but instead form a prophage associated with the bacterium genome, in effect becoming part of the bacterium. The prophage is reproduced in the same manner as the bacterial DNA when the bacterium divides, and phage may not be released for several bacterial generations. The replication cycles are known as the lytic and lysogenic pathways.

The lytic cycle is characteristic of a virulent phage and involves infection of the host, phage replication, and eventual release of progeny phage (Hendrix, 1983). The second

main cycle is the lysogenic pathway, where the viral genome integrates to a greater or lesser or extent with the bacterial genome as a prophage. In this way phage DNA will replicate only once each time the bacterium divides; a much slower process than the lytic pathway, but one which has survival value, particularly in nutrient-poor conditions (Stewart and Levin, 1984). These bacteria are referred to as lysogens, and they may have different attributes to bacteria not similarly infected, such as increased fitness (Brussow *et al*., 2004).

Bacteriophages, like bacteria, are very common in all natural environments and are directly related to the numbers of bacteria present. They are thus very common in soil and water where they play an important role in the ecology and the evolution of bacterial genomes and very importantly in the development of bacterial pathogenicity through their mediation of horizontal gene transfer as well as lysogenic conversion (Boyd and Brussow, 2002). They also influence bacterial abundance and community composition through lysis (Williamson *et al.,* 2005) and through their role as species-specific predators, phages may also help maintain microbial species diversity (John *et al*., 2002). Weinbauer and Rassoulzadegan (2004) reported that gene transfer within species may be adaptive since increasing genetic similarity and the consequent increase in genome identity should lead to a higher survival chance in stable environments. Similarly gene transfer across species and genus barriers may promote adaptation in different environments by increasing genetic diversity. Also, by moving between environments, bacteriophages can facilitate horizontal gene transfer (Breitbart and Rohwer, 2005). Bacteriophages can exert significant control on marine bacterial and phytoplankton communities, with respect to both biological production and species composition which has a major influence on matter and energy transfer pathways (Fuhrman, 1999).

Phages may contribute to the diversity of their hosts through the acquisition of prophages, which are phage genomes integrated into their host genomes. A bacterium with a prophage may acquire patho-adaptive traits such as antibiotic resistance through the acquisition of specific phage genes (Harmans *et al*., 2006) and virulence factors (Tinsley *et al.,* 2006). The presence of prophages may often account for the differences between closely related strains of bacteria (Canchaya *et al.,* 2003; 2004). The role of prophages is not limited to pathogenic bacteria but that some adaptations

of non-pathogenic bacteria strains to their ecological niche might also be mediated by prophage genomes (Brussow and Hendrix, 2002). The work of Canchaya *et al.* (2003) demonstrated that prophages contributed to a large part of strain-specific DNA, irrespective of whether the bacteria are pathogenic or non-pathogenic. Furthermore, prophages are not only a significant source of genomic divergence between strains, but also the predominant source of inter-strain phenotypic differences. In many bacteria, genome sequencing identified prophage elements as a significant proportion of the laterally acquired DNA. These elements play an important role in bacterial adaptation and have been implicated in increasing the virulence of the host and phage immunity (Rao *et al.,* 2005). In some bacterial species (e.g. *E. coli* 0157), these elements make up more than 10% of the genome (Ohnishi *et al*., 2001) with as many as 15 to 18 different prophages which account for most of the excess size of the genome of this organism (Perna *et al.,* 2001). Prophage sequences are also common in *S. enterica* where it was found in *S. enterica* serovar *Typhimurium* containing a different complement of about four or five full-size prophages (Figueroa-Bossi *et al.,* 2001).

The sequences of several genomes of pathogenic bacteria has revealed significant numbers of prophages and the genetic relatedness and diversity of the associated prophages has revealed interesting aspects in terms of phage evolution. *S. pyogenes* or group A streptococci contain large numbers of prophages (Vincent, 2007). The prophages SAI and SA2, that have been found in *S. agalactiae,* share a degree of relatedness with the *Lactococcus lactis* prophage rIt and demonstrates not only evidence for vertical phage evolution, but also lateral acquisition of diverse genetic material (Canchaya *et al*., 2003). This is echoed in other studies of prophages associated with dairy – and related, bacteria. The structural gene cluster BK5-T showed sequence similarity covering high and low DNA identity to prophages bIL286 (*Lactococcus*); Sfi21 (*Streptococcus*); adh (*Lactobacillus*) and PVL (*Staphylococcus*). Since this gradient of prophage relatedness reflects the phylogenetic relationship of their host bacteria it suggests a co-evolution of prophages with their bacterial hosts (Desiere *et al.,* 2001).

A major consequence of the acquisition of prophage DNA can be the lysogenic conversion of non-pathogenic to pathogenic bacteria. Staphylococcal enterotoxins

cause an acute food-poisoning syndrome that is the second most frequent food-borne disease in the United States. The gene for enterotoxin A is carried by several Staphylococcal prophages near their attachment sites (Betley and Mekalanos, 1985). In addition to this, *S. aureus* causes a range of diseases that include skin infections. A number of prophages have been found in clinical isolates of *S. aureus*. The sequencing of the prophage DNA revealed the carriage of several putative toxin genes. The five major types of *S. aureus* prophages which include phiMu50A and phiMu50B are prophages extracted from the sequenced Mu50 *S. aureus* strain, while several other types have been induced from uncharacterised *S. aureus* strains (Canchaya *et al*., 2003).

Lysogenization of different Clostridia by some bacteriophages has different effects on the host phenotype, some of which can convert them from the non-toxigenic form into toxigenic isolates (Eklund *et al*., 1972). Clostridia are widely disseminated in soil and lakes but are also found in the intestinal flora of humans and animal. A *C. botulinum* is the isolate that produces botulinum toxin, which causes food-poisoning. The *C. perfrigens* phage phi3626 showed the typical genome organization of Sfi21-like Siphoviridae. The sequenced *C. acetobutylicum* strain used in industrial fermentation (Nolling *et al*., 2001) contained two prophages (Prophage 1 and Prophage 2) and *C. tetani* strain E88 contains three prophages, phiCT1, phiCT2, and phiCT3 (Canchaya *et al*., 2003).

Another group of bacteria that have become pathogenic as a result of lysogenic conversion are the Shiga-toxin producing *E. coli* (STEC) strains which represent serious emerging food-borne pathogens and carry prophage content up to 20% of the genome. The two sequenced O157 strains, EDL 933 and Sakai, are very similar with respect to their bacterial DNA but differ primarily in their prophage content (Ohnishi *et al*., 2001; Perna *et al*., 2001). In EDL 933, 12 prophage sequences were identified, as opposed to Sakai which contains 18 prophage genome elements. Of these prophages only the Shiga- toxin-converting phage 933W can produce infectious phage particles (Plunkett *et al.,* 1999). In this EDL 933 strain there are nine defective lambda-like prophages which have large regions of high DNA sequence identity to phage lambda and to prophages from the Sakai strain (Canchaya *et al*., 2003).

Not only do phages have a major impact on the diversity of their bacterial hosts, but they are also extremely diverse. James *et al.* (2006) have examined the genetic diversity of the genomes of five related T4-like bacteriophages; the *Escherichia coli*-associated phages RB43, RB49 and RB69; the *Aeromonas salmonicida*-associated phage 44RR2.8t and the *Aeromonas hydrophila* phage Aeh1. Their result suggests that the evolution of the T4-like phages has drawn on a highly diverged pool of genes in the microbial world. Furthermore, 62 bacteriophages were isolated on eight indigenous bacteria from a Pacific Ocean station and in this study they demonstrated that the genetic diversity of bacteriophages in the Ocean is far greater than that of their bacterial hosts (Jiang *et al*., 2003).

1.2.3.2. Mycobacteriophages

Mycobacteriophages exhibit extraordinary diversity and may be found in all natural ecosystems that have been colonized by mycobacteria (McNerney and Traoré, 2005). Several of these phages, which are predominantly virulent (or lytic), have been isolated directly from the environment. Lytic mycobacteriophages able to infect fast-growing mycobacteria such as *M. smegmatis* were first isolated from soil and leaf mould (Gardner and Weiser, 1947). Later, mycobacteriophages able to infect slow-growing mycobacteria, such as members of the *M. tuberculosis* complex, were also discovered (Froman *et al*., 1954). Penso and Ortali (1949) isolated five phages which were active against different saprophytic mycobacterial species. Recently Bxz2 was isolated from environmental samples from the Bronx Zoo by the William R. Jacobs laboratory at the Albert Einstein College of Medicine, New York (Pedulla *et al*., 2003). Sunhee *et al.* (2004) reported the isolation and characterization of Bxzl phage, from soil at a wildlife Preservation Park, while Pham *et al.* (2007) isolated mycobacteriophage Tweety from a moist soil sample from an urban grass lawn in the Oakland district near the University of Pittsburgh (PA, USA).

Mycobacteriophages have also been extensively induced from lysogenic mycobacteria. Doke (1960) isolated the temperate phage L5 from cultures of *M. smegmatis* while its lytic counterpart, Mycobacteriophage D29, was isolated from soil by Froman *et al.* (1954). The temperate phage TM4 was isolated from cultures of *M.*

avium following treatment with the DNA damaging agent mitomycin C (Timme and Brennan, 1984).

Mycobacteriophages have varying host-ranges as is shown in Table 3. Mycobacteriophage L5 is a temperate phage that infects both fast- and slow-growing mycobacteria (Fullner and Hatfull, 1997) and mycobacteriophage D29 is a lytic phage which is able to infect slow-growing pathogenic strains such as *M. tuberculosis*, *M. ulcerans* and fast-growing *M. smegmatis* (Froman *et al.,* 1954). Redmond and Cater (1960) discovered that mycobacteriophage DS6A was specific for members of the *M. tuberculosis* complex. However, the infection of mycobacteriophages required favorable conditions such as optimal $CaCl_2$ concentrations which are very important for a number of mycobacteriophages and other phages as it often affects attachment. It is possible that different concentrations may have a significant effect on the host range of some phages (Rybniker *et al*., 2006).

Recently a significant number of phages have been isolated from the environment and sequenced, which has offered important insights into the nature of phage diversity and evolution. Thirty different phages from various geographical and environmental locations have been isolated using *M. smegmatis* as the sensitive host strain (Pedulla *et al*., 2003; Hatfull *et al*., 2006). The comparison of these genome sequences with previously characterised mycobacteriophages revealed some interesting findings including the fact that the genomes were considerably diverse with high proportion of unique genes almost 50% that are unrelated to those of other mycobacteriophages or any other previously sequenced organism; three quarters of the remaining 50% of genes match only other mycobacteriophage genes such that 87% of all mycobacteriophage genes are unrelated to the gene pool outside of the mycobacteriophages (Pedulla *et al*., 2003). Some of the phages could be clustered together in terms of sequence similarity, for example the previously characterised phage Bxz2, which has sequence similarity with L5, D29, and Bxb1. Some of these recently isolated phages Che9d, Che8, and Omega do share limited segments of nucleotide sequence similarity. The lengths of complete genome sequences of these mycobacteriophages vary considerably, but with the exception of Bxz2 (50.9 kbp), all are larger than the previously sequenced mycobacteriophages and the sizes range from 49.1 to 156 kbp which is within the expected range of overall phage genome

sizes (Pedulla *et al*., 2003). These genome comparisons demonstrated how genes are exchanged between mycobacteriophages – in a mechanism that differs little from other characterised phages (Hendrix, 2002). They showed that gene exchange occurs more frequently between the mycobacteriophage populations than it does between the mycobacteriophage and the host genomes. Acquisition of genes by the mycobacteriophage pool is relatively rare compared to the rate at which genetic content disseminates within the population. The data strongly suggest that illegitimate recombination plays an important role in this process, recombining viral and non-viral DNA molecules in a sequence-independent manner. Horizontal exchange of sequences occurs most frequently among closely related phages, but it also extends across the entire global population at lower frequency (Hendrix, 2002).

Table 3. Host range of mycobacteriophages

	Mycobacteria											
Phages												
	M. smegmatis	*M. fortuitum*	*M. phlei*	*M. tuberculosis*	*M. bovis*	*M. avium*	*M. kansasii*	*M. scrofulaceum*	*M. ulcerans*	*M .marinum*	*M. chelonae*	References
Barnyard	+	-	-	-	-	-	-	-	-	-	-	Rybniker *et al.*, 2006
DS6A	+	ND	ND	+	+	-	ND	ND	ND	ND	ND	Redmond and Cater, 1960
Cjw1	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Pedulla *et al*., 2003
Bxz2	+	+	ND	+	+	+	ND	+	+	-	+	Rybniker *et al.*, 2006
Che8	+	-	ND	-	-	-	ND	-	-	-	-	"
D29	+	-	ND	+	+	+	ND	+	+	-	-	"
L5	+	+	ND	+	+	+	ND	+	+	-	+	"
Omega	+	-	ND	-	-	-	ND	-	-	-	-	"
PBII	+		ND	-	-	+	ND	+	-	-	-	"
PG2	+	+	ND	-	-	-	ND	+	-	-	+	"
Rosebush	+	-	ND	-	-	-	ND	-	-	-	-	"
SBI	+	-	ND	-	-	-	ND	-	-	-	-	"
Cooper	+	-	ND	-	-	-	ND	-	-	-	-	"
TM4	+	-	ND	+	ND	+	ND	-	+	-	-	Rybniker *et al*., 2006. Timme and Brennan, 1984
TM9	+	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	Timme and Brennan, 1984
TM10	+	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	"
Bxb1	+	ND	ND	ND	-	-	-	ND	ND	ND	ND	Mediavilla *et al*., 2000

Abbreviations for different mycobacteria in the Table are as follows: += infects, - = does not infect, ND= Not determine

As mentioned previously prophages are particularly prominent in bacterial pathogens, since they frequently encode virulence genes and are major contributors to the genetic individuality of the strains (Canchaya *et al*., 2004). Unlike in *E. coli* and other pathogenic bacteria, there are relatively few prophages in mycobacteria and the identified prophages are prophage-like elements which may be associated with phenotypic consequence (Bibb and Hatfull, 2002). Several possible roles can be contemplated for these elements, for example, one or more of the encoded genes could influence the growth rate, metabolic requirements or pathogenicity of the host. Evidence has been presented by others that prophage-like elements may make a significant contribution to the virulence potential of a bacterial host including *S. enterica* (Boyd and Brussow, 2002), such prophages can pick up specific virulence associated genes such as *sopE* encoded by which affects tissue-specific attachment (Mirold *et al*., 1999). Prophage-like elements play a critical role in the generation of genetic diversity within *S. enterica* (Thomson *et al*., 2003). The genomes of *M. tuberculosis* H37Rv and CDC1551 have been found to contain two such prophage-like elements, ΦRv1 and ΦRv2 (Bibb and Hatfull, 2002). The avirulent strains of *M. bovis* BCG have no prophage elements, while all clinical isolates of *M. tuberculosis* have at least one copy of either ΦRv1 or ΦRv2, suggesting that it could play an indirect role in the physiology of *M. tuberculosis*, by conferring the ability to form virus-like particles that may be capable of generalized transduction (Lang and Beatty, 2000).

1.3. Aims, research hypothesis and objectives

Environmental mycobacteria can be found in diverse environmental sources and most of them have a saprophytic lifestyle. However, some have the ability to infect animals, birds and humans. Because these organisms are widespread in the environment, and there is little evidence that person to person transmission is common, there is an implicit assumption that environmental mycobacterial infections derive from environmental sources including soil, water, food, and dust (Falkinham, 1996). It has been suggested that mycobacteria associated phages may make an important contribution to the evolution of pathogenic mycobacteria (Pedulla *et al*., 2003). Bacteriophages, like bacteria, are very common in the environment. Very little

is known about the diversity and association of mycobacteriophages with environmental mycobacteria. It is therefore important to assess whether environmental mycobacteria isolated in South Africa are associated with, or contain inducible phages. This study is therefore focused on the isolation of inducible mycobacteriophages from isolates of environmental mycobacteria and the isolation of mycobacteriophages directly from soil samples that may be associated with environmental mycobacteria.

1.3.1. Main objective

To isolate environmental mycobacteria and associated mycobacteriophages from soil, water and surfaces collected at different locations.

1.3.2. The specific objectives will then be as follows:

- Isolation of mycobacteria;
- Identification of mycobacteria;
- Isolation of inducible phage/s from mycobacterial isolates;
- Isolation of mycobacteriophages directly from soil samples from which isolates of mycobacteria have been obtained;
- Characterization of mycobacteriophages based on morphological and molecular techniques

CHAPTER 2 - ISOLATION AND CHARACTERISATION OF ENVIRONMENTAL MYCOBACTERIA

2.1. Introduction

The genus *Mycobacterium* contains more than 90 species (http://www.bacterio.cict.fr/m/mycobacterium.html) including organisms that cause serious human and animal diseases. Isolation of mycobacteria from environmental samples is complicated by the presence of other microbes in the environment and all mycobacteria are not equally resistant to the different decontamination procedures. The isolation of mycobacteria from soil and water involves three steps: elution from particulate matter, concentration and decontamination (Songer, 1981; Conville *et al*., 1995). Different modifications have been applied by different workers in an attempt to optimize the diversity and purity of mycobacteria isolated. Some of these modifications are described below. The decontamination of environmental material is a key concern and the innate alkali resistance of many mycobacteria allows the use of NaOH to selectively remove contaminating species other than mycobacteria. Kamala *et al*. (1994) compared different methods for isolating mycobacteria from samples of soil and water. They found that decontamination with 1% NaOH and 3% sodium lauryl sulphate was significantly superior to those methods using a higher concentration of NaOH and other decontamination procedures. However, other approaches to decontamination may also be considered. Yamamura and Harayama (2007), for example, showed that the non-ionic detergent SDS may also be used for decontamination (Parashar *et al.,* 2004). Additional selection is also achieved by the use of an appropriate growth media. Standard growth media such as Lowenstein-Jensen (LJ) agar, used for cultivation of members of the *M. tuberculosis* complex, have a pH of 7.0, which lies well above the pH optimum of 5.4 to 6.5 found for many environmental mycobacteria (Portaels and Pattyn, 1982). Portaels *et al.* (1988) compared different culture media for the isolation of mycobacteria from soil samples and obtained the best results by pre-incubation of the soil samples in tryptic soy broth (TSB) followed by decontamination with malachite green, cycloheximide and NaOH to suppress fungal overgrowth and other microbial overgrowth. This was then cultivated on Ogawa medium. Lowenstein Jensen media did give higher mycobacteria yields than inoculation onto Falkinham's selective medium, which is a minimal agar

medium containing Tween 80 as the sole carbon source (George and Falkinham, 1986). Novel approaches using olive oil as a carbon source (Yamamura and Harayama, 2007) for the selective isolation of mycobacteria have proved successful for isolating some mycobacteria since the presence of hydrophobic mycolic acids in the cell wall makes the surfaces of mycobacteria highly hydrophobic and therefore gives it an affinity for this hydrophobic carbon source.

The identification and methods of classification of mycobacteria species has greatly benefited from the development of molecular biological tools dependent on the characterisation – at different levels of resolution (restriction fragment length polymorphism [RFLP]; sequencing) - of one or several appropriate genes. These are being employed because they yield quick and generally unequivocal results (Kolbert and Persing, 1999). These methods allow a universal standardization of results which facilitate comparisons between different laboratories (Devulder *et al*., 2005). These approaches have had a profound effect on identification of environmental mycobacteria in the clinical setting (Gomila *et al*., 2007). In terms of which genes are considered for the specific typing of mycobacteria, several have been considered. Stackebrandt and Goebel (1994) proposed that isolates where the 16S rRNA sequences are less than 97% identical may be classified as different species. Since then, sequencing and analysis of 16S rRNA genes have been preeminent in the advancement of bacterial taxonomy (Stackebrandt and Goebel, 1994; Von Wintzingerode *et al.,* 1997). In the genus *Mycobacterium* however, the 16S rRNA genes of the different species differ by only 5 to 15 base pairs (Kirschner *et al*., 1992; Kusumoki and Ezaki, 1992). The high conservation of the 16S rRNA gene has limited its utility in the species identification of mycobacteria. The 65 kD heat shock protein gene (*hsp65*), a less conserved gene that appears ubiquitous among mycobacteria, has proven to be a useful tool, not only as a genus-specific marker, but also in the high resolution identification of most mycobacterial species (Adékambi and Drancourt, 2004; McNabb *et al.,* 2004). In addition to *hsp65*, the RNA polymerase β-subunit gene (*rpoB*) (Ko *et al*., 2002; Adékambi *et al*., 2003) has also been used for species-specific identification of mycobacteria. Given the utility of the *hsp65* gene for phylogenetic reconstruction and species identification (Telenti *et al.,* 1993) it was used in the present study to characterize the different mycobacteria isolates.

2.2. Aims and Objectives

In order to assess the phages associated with mycobacteria it was first necessary to isolate environmental mycobacteria from various sources. The objective of this chapter was therefore to isolate environmental mycobacteria from different environmental sources and characterise the isolates using genetic techniques which included a genus-specific PCR amplification of the *hsp65* gene and then resolving these isolates to the species level by sequencing of this gene product and subjecting the data to phylogenetic analysis.

2.3. Materials and Methods

2.3.1. Sampling

A total of 25 soil samples were obtained from the University of Johannesburg Mycotoxin Laboratory, all those samples were collected from Mpumalanga and Limpopo rural areas; six soil samples were collected from the Wits University ecological gardens and two water samples from west campus lake; five surfaces (associated with tap water) samples were collected from Yeoville Johannesburg in residential accommodations; and 10 river water samples from Bedfordview in Johannesburg were also collected. All samples were collected in sterile, Polypropylene Falcon tubes (Lasec) at the ambient temperature. From each site, one to four samples were collected. Samples, stored at 4^{o}C, were processed within two weeks. Only one isolate was recovered for any sample to avoid sib-selection.

2.3.2. Recovery of mycobacteria from soil and water samples and growth on solid media

Mycobacteria were isolated from soil and water samples using a method adapted from Parashar *et al*. (2004).

Soil samples. Five grams of soil were suspended in double-distilled autoclaved water (D/W), in 50ml Polypropylene Falcon centrifuge tubes (Lasec). This was manually shaken for 2 min. and the suspension centrifuged at 600×g for 5 min. to pellet the soil particles. The turbid supernatant (10ml) was transferred into another centrifuge tube and centrifuged at 8000×g for 15 min. at room temperature to pellet the cells.

Water samples. Water samples were centrifuged at 8000×g for 15 min. at room temperature.

Surface-associated. The material adhering the interior surface of either a pipe or a water meter was collected by scraping the surface with a sterile cotton bud and suspending this in 10 ml of sterile distilled water and processed further as described for water samples.

The pelleted material from soil, water and surfaces was resuspended in a solution of 3% SDS, 4% NaOH for 15 min. to eliminate non-mycobacteria species (decontamination). The suspensions were then centrifuged at 8000×g to recover mycobacteria cells and 0.5ml of double-distilled autoclaved water (D/W) was added to the pelleted cells and 0.1ml of this was plated on 7H10 agar (Difco) plates supplemented with 1mM $CaCl_2$, 1mM $MgSO_4$, 0.3% Glucose, and incubated at 37^{o}C. The plates were examined daily for one to two weeks for colony development. Single colonies were picked and subjected to ZN staining (section 2.3.3). Isolates staining positive with the ZN stain were sub-cultured twice to ensure the purity of the isolates.

2.3.3. Biochemical identification of mycobacteria

Individual colonies were checked for acid and alcohol fastness by Ziehl-Neelsen staining, in which mycobacteria stain bright red in contrast to a blue background when visualized using a light microscope (IRI Research Instrumentation, Nikon Japan) at 1000x magnification (Abe, 2003). Briefly, heat fix impression smears by passing slides over flame three or four times in biohazard cabinet; place slides on staining rack and cover it with carbolfuchsin stain and heat steam for 5 min. After rinsing thoroughly with tap water, the sample was subjected to decolorization by rinsing with acid alcohol for 2 min. and then rinse again thoroughly with tap water before counterstaining with malachite green for 1 min. and follow with last step of rinsing thoroughly with tap water and leave to air dry.

2.3.4. Liquid culturing of mycobacteria

Single colonies from 7H10 plates were inoculated into 5ml of Middlebrook 7H9 broth (Difco) supplemented with 1mM $CaCl_2$, 1mM $MgSO_4$, 0.3% Glucose as well as 0.06%Tween 80, which prevents clumping of mycobacteria (Meyers *et al*, 1998; Parish and Stoker, 1998) and grown for several days – depending on the specific growth properties of the different isolates. Once these cultures had reached an optical density (OD_{600nm}) of at least 2.0, they were inoculated (1:100) into 20 or 50ml cultures of 7H9 media, supplemented as previously mentioned and were then periodically sampled over a 24h period and plated on 7H10 plates, following serial dilution in Tween 80 (0.06%)-supplemented 7H9 media.

The growth rate of the different isolates was determined by producing two independent growth curves as described above. The exponential growth phase was determined by doing a regression analysis of the points in the growth curve which were in the linear range (determined visually) from a semi-log plot. The growth rate constant (μ) was determined using the following formula:

$$\mu = [(\log_{10}N_1 - \log_{10}N0)]2.303/(t_1 - t_0)$$

The generation time (g) was calculated as follows:

$$g = \log_{10}N_1 - \log_{10}N_0/\log_{10}2$$

Where N_1 = cfu/ml at a selected time (t_1) in the exponential phase; N_0 = cfu/ml at an initial time (t_0) in the exponential phase of growth.

2.3.5. Mycobacteria identification with *hsp*65 genus-specific PCR.

2.3.5.1. DNA Sample preparation

The isolates were grown in liquid 7H9 media as described in (section 2.3.4) until an OD_{600nm} of approximately 1.5 was reached. Nucleic acids were extracted by lysing bacterial cells. This was done by centrifuging 500μl of culture at 13,000×g for 10

min. and collecting the pellet, which was resuspended in 0.1 ml of PCR grade water (Adcock Ingram) and boiled for 10 min. to lyse the cells. This was briefly centrifuged (1 min.; 13,000×g) to remove cell debris and following this the supernatant was transferred to a sterile Eppendorf tube. A volume of 5µl was used as a template in the PCR reaction. This method was modified from Chang *et al*. (2002).

2.3.5.2. Genus-specific PCR Amplification of *hsp65*

Amplification of the *hsp65* gene of the putative *Mycobacterium* isolates was carried out using mycobacteria-specific primers targeted to the *hsp65* gene for the identification of mycobacteria. The primers used for amplification were *hsp*-forward (5'ACCAACGATGGTGTGTCCAT3') and *hsp*-reverse (5'CTTGTCGAACCGCATACCCT3') (Telenti *et al*., 1993). The PCR reaction mixture (50µl) contained 5µl of mycobacteria extracted DNA (see section 2.3.5.1), *hsp*-forward and reverse primers (100µM each), 5U *Taq* DNA polymerase (Bioline), 10× NH_4 buffer, 50mM $MgCl_2$ and 100mM dNTP mix. The reaction was subjected to thermal cycling which included an initial denaturation step of 95^{o}C for 11 min. followed by a primer annealing step of 59^{o}C for 45s and a polymerization extension step of 72^{o}C for 1 min. This was followed by thirty five cycles of 30s at 95^{o}C, 45s at 59^{o}C and 45s at 72^{o}C. A final cycle of 1 min. at 95^{o}C, 45s at 59^{o}C and 10 min. at 72^{o}C was completed. A fraction of the samples were analyzed by agarose gel electrophoresis (section 2.3.5.3).

2.3.5.3. Agarose gel electrophoresis

After PCR amplification, 2µl of gel loading buffer (Fermentas) was added directly to 8µl of the PCR product which was loaded onto a 1% agarose gel supplemented with 0.002% ethidium bromide and electrophoresis was carried out in running buffer (1xTAE: 0.04 M Tris acetate and 0.001M EDTA, pH 8) at 100V. Fragments were visualized under UV light. The gels were viewed and images digitally captured using a UV gel documentation imaging system (UVP BioDoc-It, Model LM-26E).

2.3.5.4. Sequencing and Bioinformatics Analysis

PCR products of all mycobacteria isolates were sequenced on both strands using the genus-specific *hsp65* primers (section 2.3.5.2). The sequencing was outsourced to Inqaba Biotechnological Industries. Sequence chromatographs were visually inspected for errors and internally verified by aligning both strands (following reverse complementation of one of the strands) using Chromas. The verified sequences were subjected to an homology-based BLAST analysis against the NCBI GenBank database. Multiple alignments of the nucleotide sequences were done with specific algorithms contained in the DNAMAN suite of DNA analysis software (version 4.15) and the genetic distance tree was constructed based on the derived *hsp65* nucleotide sequences.

2.4. Results

2.4.1. Isolation of mycobacteria

In order to isolate environmental mycobacteria, we looked at different environmental sources including soil, water, and various water-associated surfaces from selected sites in Johannesburg, Mpumalanga and Limpopo. Overall, out of 48 environmental samples collected from different sources, 11 samples yielded acid-fast bacilli as determined by ZN staining (see Table 4 below). Soil yielded the highest proportion of acid fast isolates (6 out of 24). Water (2/11) and surfaces (3/13) yielded similar frequencies of acid-fast isolates.

2.4.2. Genus-specific PCR analysis of acid-fast isolates

To confirm that the ZN-positive samples were mycobacteria, genus-specific PCR amplification of the *Mycobacterium*-specific *hsp65* gene, which should yield 439bp amplicon (Telenti *et al*., 1993), was done. As can be seen in Fig. 1, all isolates, including the laboratory strain *M. smegmatis* mc^2155 (Fig. 1A, lane 8; Fig. 1B, lane 9) yielded an amplicon of the anticipated size. Even though isolate S3 (Fig. 1B, lane 5) failed to amplify, it was amplified following a separate DNA extraction (Fig. 1A, lane 6). These results confirmed that the ZN-positive amplicons were mycobacteria.

Table 4. Recovery of ZN-positive isolates from different sources

No. of ZN-positive samples / total no. of samples				
Site	**Soil**	**Water**	**Surfaces**	**Total**
Limpopo	5/10	-	-	10
Mpumalanga	1/8	-	-	8
Jhb Yeoville	-	0/4	3/13	17
Jhb Wits	0/6	2/7	-	13
Total	6/24	2/11	3/13	11/48

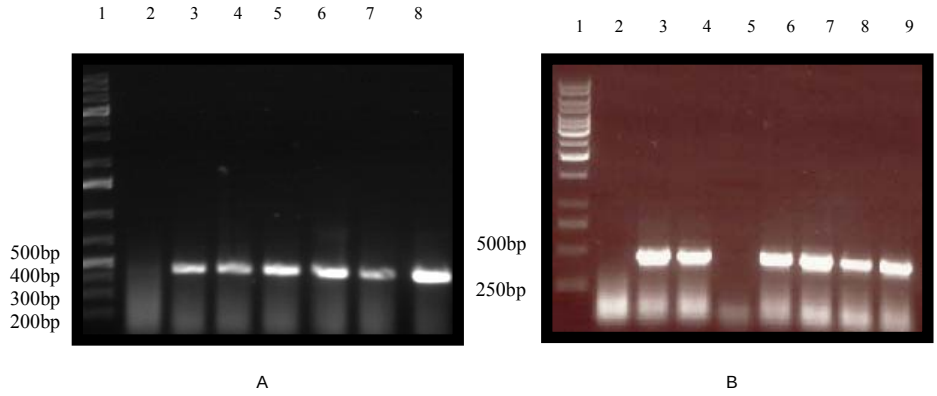


Figure 1. A and B. **Agarose gel of PCR amplified isolate DNA with *hsp65* mycobacteria genus-specific primers**. **A**. Lane 1: DNA size marker (O'RangeRuler 1kb, Fermentas); lane 2: contains the no DNA control; lane 3 to 8 are the different isolate DNA: Lane 3: BI; lane 4: B2; lane 5: B3; lane 6: S3; lane 7: S9 and Lane 8: *M. smegmatis* mc^2155. **B**. Lane 1: DNA size marker (Hyperladder 1, Bioline); lane 2: no DNA control; lanes 3 to 8 contain isolate DNA. Lane 3: S1; lane 4: S2; lane 5: S3; lane 6: SB; lane 7: S7; lane 8: S8 and lane 9: *M. smegmatis* mc^2155. PCR amplification was based on *Mycobacterium* genus-specific *hsp65* gene which is expected to yield a 439bp amplicon (Telenti *et al.*, 1993).

2.4.3. *hsp65* gene sequencing and phylogenetic analysis

In order to estimate the diversity of the isolated mycobacteria, nucleotide sequencing and a phylogenetic comparison of isolate-derived PCR products was done in comparison to *M. smegmatis* mc^2155 and other well characterised mycobacteria. As can be seen from Fig. 2 the 10 isolates characterised by *hsp65* sequence analysis could be clustered into four groups: (I) *M. aubagnense*-like; (II) *M. mucogenicum*-like; (III) *M. smegmatis*-like; (IV) *M. fortuitum*-like. The overall sequence similarity

for the *hsp65* sequence data was 98% for group I, 99% group II, 98% group III and group IV 99%. In these experiments soil yielded the highest diversity of mycobacteria, but this may be because soil yielded the highest number of sequenced isolates (7/10). It is also interesting to note, that even though only a single isolate was recovered from any sample, surfaces displayed a clustering of isolates in terms of homology to different isolates of *M. mucogenicum*.

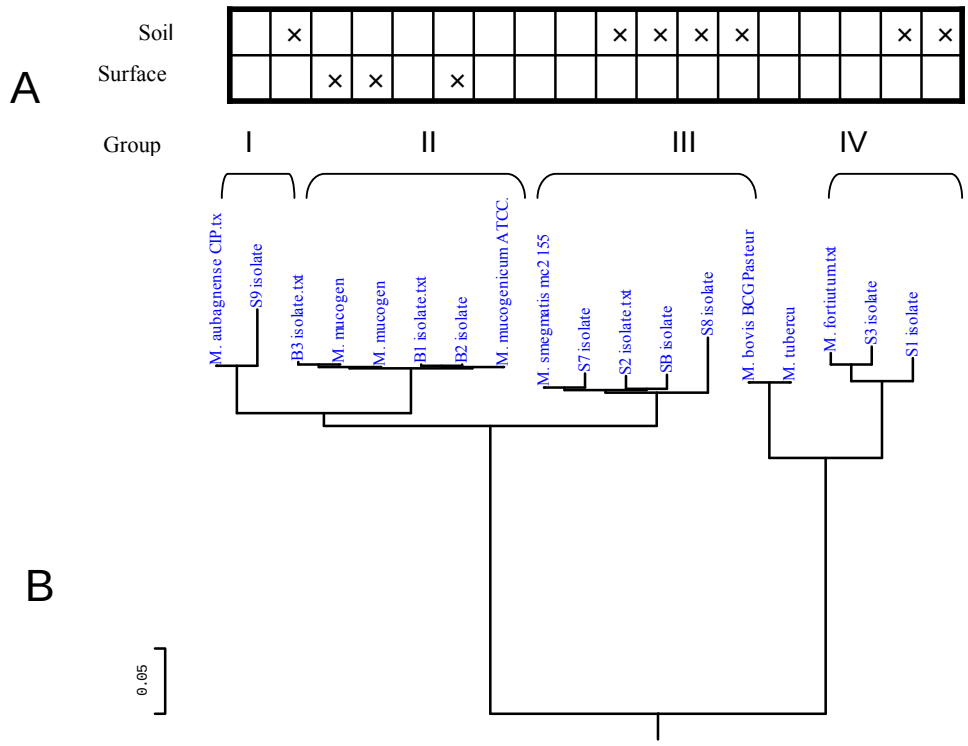


Figure 2. DNA sequence alignment of the different mycobacteria isolates. A. Table of mycobacteria source (indicated with ×), shown above its position in the genetic distance tree, which is indicated in B. Homology is based on sequence comparison of the 439bp region of *hsp65* gene sequence from the different mycobacteria isolates. The comparisons were performed together with other published mycobacteria sequences using the suite of programmes contained within the DNAMAN software package version 4.15. The bar at bottom left indicates 0.05 (5%) nucleotide sequence difference.

2.4.4. Liquid culture analysis of mycobacteria isolates

Reports from the literature suggest that all the isolates obtained should be fast-growing mycobacteria. The most commonly occurring *Mycobacterium* in the environment is probably *M. fortuitum*, as it is often the major *Mycobacterium* species

(40-80%) isolated from soil samples (Tsukamura, 1984). Strains of the rapidly growing species *M. smegmatis, M. aurum* and *M. agri* have also been isolated frequently from soil samples (Tsukamura, 1984). A *M. aubagnense* (Adékambi *et al*., 2006a); *M. mucogenicum* (Gomila *et al*., 2007; Band *et al*., 1982).

To confirm this growth analysis of the mycobacteria were determined. Pure cultures of the mycobacteria isolates were therefore grown in tween 80-supplemented Middlebrook 7H9 media as described in the materials and methods and incubated at 37^{o}C for 24h and periodically sampled for colony forming unit (cfu) analysis. We were unable to develop growth curves for the *M. mucogenicum*-like mycobacteria isolates (B1, B2 and B3) obtained from surfaces due to an unknown associated yellow substance which prevented us from counting colonies. As can be seen from Table 5 all isolates could be reasonably classified as fast-growers, as their generation times were similar to the fast-growing *M. smegmatis* mc^{2}155 control (3.1h). The other *M. smegmatis*-like isolates all displayed a similar generation time ranging between 3.1 and 3.4h. *M. fortuitum*-like isolates S9 and S1 displayed the widest range of growth rates, which were 2.7h and 4.5h respectively.

Table 5. Growth rate analysis of different mycobacteria isolates.

Isolate	Group[2]	Growth rate constant, μ (hour^{-1})	Generation time, g (hours)
M. smegmatis mc^{2}155[1]	III	0.9±0.44	3.1±0.64
S2		0.9±0.05	3.3±0.56
SB		0.8±0.0	3.4±0.02
S8		1.0±0.2	3.4±0.42
S7		0.9±0.2	3.3±2.12
S9	I	0.97±0.1	2.8±0.28
S1	IV	1.55±0.2	4.5±0.54
S3		0.76±0.1	2.7±0.31

[1] Laboratory strain; [2] Group designations according to *hsp65* sequence similarity: Group I, *M. aubugnese*-like; II, *M. mucogenicum*-like; III, *M. smegmatis*-like; IV, *M. fortuitum*-like. Data obtained from two independent growth curves (see appendix C). The growth rate of *M. smegmatis* has been also demonstrated previously (Güthlein *et al*., 2008; Gadagkar and Gopinathan, 1980).

2.5. Discussion and Conclusion

In this study, we tried to isolate and identify mycobacteria from environmental samples including soils and water from different places in South Africa, which will then be used to see whether there are associated prophages. In total, 10 mycobacteria isolates were recovered from four different sites (Table 4). The growth analysis suggested that all mycobacteria isolates were fast-growing mycobacteria (see Appendix C). Genetic and biochemical analysis was used for species identification. Sequencing of the *hsp65* 439bp fragment of our isolates indicated that all our isolates have high sequence homology with isolates identifiable in GenBank.

The isolates obtained were shown in our *hsp65* genetic distance analysis to cluster with previously identified mycobacteria: *M. smegmatis, M. fortuitum, M. aubagnense* and *M. mucogenicum* strains. *Mycobacterium fortuitum* clustered with S3 and S1 mycobacteria isolates; Mycobacteria isolates B1, B2, and B3 clustered with *M. mucogenicum*; the S9 mycobacteria isolate clustered with *M. aubagnense*; and the four mycobacteria isolates (S2, SB, S7, and S8) formed a cluster with *M. smegmatis*. *Mycobacterium tuberculosis* and *M. bovis* were included in the analysis as reference strains and these slow-growing mycobacteria formed a separate cluster from our fast growing mycobacteria. Although our isolates revealed high genetic identity to well known mycobacteria sequences captured in GenBank it still remains a possibility that they might be genetically different. Adékambi and Drancourt, (2004) demonstrated that 16S rRNA and *hsp65* gene sequences could not alone resolve the phylogenetic relationships between currently recognized rapidly growing *Mycobacterium* species due to the failure of 16S rRNA and *hsp65* gene sequences to discriminate *M. houstonense, M. senegalense* and *M. farcinogenes* or *M. mucogenicum* ATCC 49650 from *M. mucogenicum* ATCC 49651. The percentage of similarity of 19 rapidly growing mycobacteria examined was 95.5%-100%, explaining the poor phylogenetic information obtained from those sequencies (Adékambi and Drancourt, 2004). However, *rpoB* gene sequence clearly separated *M. mucogenicum* ATCC 49649 from *M. mucogenicum* ATCC 49650 and *M. mucogenicum* ATCC 49651 despite the fact that they belong to the same species (Springer *et al*., 1995); and *M. senegalense* and *M. farcinogenes* (Adékambi and Dancourt, 2004). Drancourt *et al*. (2000) suggested that ≥ 99% similarity regarding 16S rDNA sequence comparison between two isolates

defines a similar species. Although acceptable for other bacteria, this definition does not apply to mycobacteria (Ferdinant *et al.,* 2004). Indeed, the latter are characterized by a unique genetic relatedness, e.g. two strains with a 99% similarity in their 16S rDNA may or may not belong to the same species (Tortoli *et al*., 2001). For example, according to host-specifity, *M. tuberculosis* and *M. bovis* are considered as distinct species, though at the molecular level these may be defined as subspecies of *M. tuberculosis* complex (Rastogi *et al*., 2001).

Our growth rate analysis indicated that all of our mycobacteria isolates were rapidly growing mycobacteria. The detection of only rapid growing mycobacteria could be due to the procedure used for their isolation. In contrast to the method used by Parashar *et al*. (2004) slightly modified, the samples were decontaminated only with SDS and NaOH, and not with cetrimide which should be added to remove all contaminants present in the samples, and the duration of the incubation period of the decontaminants in the samples when isolating slow growing mycobacteria was not applied, a procedure which is commonly used when isolating mycobacteria. Because of their relatively slow growth rate and susceptibility to overgrowth by faster-growing organisms on solid media (Wang *et al*., 2006); this decontamination step might result in a loss of sensitive mycobacteria and bias in their recovery (Hartmans and DeBont, 1992).

The environmental mycobacteria were found from different sources and the trend seemed to suggest that they were more likely to be isolated from soil, but were also to a lesser degree present in tap drinking water and associated surfaces (Table 4). There are several possible explanations for these observations. Kamala *et al*. (1994) demonstrated that the *M. fortuitum* complex is predominant in soil. The glycopeptidolipids present in the outer most layers of the cell walls of mycobacteria play an important role in surface colonization and biofilm formation (Martinez and Torellos, 1999; Recht and Kolter, 2001). The biofilms represent an important risk of contamination for water distribution systems (Schulze-Röbbecke *et al.,* 1992). Schulze-Röbbecke *et al.* (1992) demonstrated that organic substances such as plastics and rubber were usually colonized by larger numbers of mycobacteria than inorganic substances such as copper and glass. The results obtained in this study confirmed previous findings, that environmental mycobacteria are inhabitant of a wide range of

environmental sources. *Mycobacterium mucogenicum* rapidly growing mycobacteria frequently isolated from tap water (Adékambi *et al*., 2006b); forms a distinct group closely related to *M. abscessus* and *M. fortuitum* groups among rapidly growing mycobacteria (Adékambi and Dancourt, 2004). Bland *et al.* (2005) reported that *M. fortuitum* complex were the most commonly rapidly growing mycobacteria isolated from the freshwater river and biofilm samples (Shulze-Röbbecke and Buchholtz, 1992).

The isolation and characterization of mycobacteria from new settings may lead to isolation of new prophages and phages and enable us to have knowledge and understanding of the interaction between phages and their host mycobacteria.

CHAPTER THREE - ISOLATION AND CHARACTERIZATION OF MYCOBACTERIA ASSOCIATED PHAGES

3.1. Introduction

Mycobacteriophages have proved to be extremely useful tools for the development of mycobacterial genetics, as well as revealing novel aspects of viral evolution (Hendrix *et al*., 1999). Temperate bacteriophages possess a specific mode of existence, able either to lyse sensitive cells or to form stable lysogens in which the phage genome is propagated as a prophage by bacterium with the phage lytic functions repressed by one or several phage repressor proteins. Cells may undergo several rounds of division but occasionally one will spontaneously lyse and liberate progeny phage. The search for temperate phages that are in lysogenic association with their host cells can be performed without any treatment that may stimulate induction since many phages are spontaneously induced (Audurier *et al*., 1977); or after induction by means of different physical or chemical treatments, generally ultraviolet irradiation and mitomycin C treatment which, at least in some characterised phage-host systems, may lead to phage lytic development (Loessner *et al.,* 1991; Estela *et al.*, 1992).

Rieber and Imaeda (1969) reported that the induction of mycobacteriophages from *M. bovis* BCG and other mycobacterial species have revealed that the mitomycin C induced particles are characterized by showing abnormally long tail structures, in comparison with the spontaneously released phages from lysogenic strains.

Various methods used for the assessments of bacteriophage abundance in different environmental sources are based on determination of the plaque forming units (pfu) produced by the phage on sensitive indicator cells (Yales *et al*., 1990). However, several approaches to determine phage abundance do not depend on the direct isolation of the phage (i.e. the requirement for a sensitive host strain), but on the visual identification of putative phage and are based on microscopic enumeration of optically active viruses (Taverner and Connor, 1992). Recently Epifluorescent Microscopy (EFM) was used to estimate virus abundances in different soils by direct counting of virus-like particles. The principle of this method resides in the ability of SYBR Green I dye to penetrate bacteriophage capsids and intercalate in the nucleic acid, displaying a higher affinity for dsDNA than other nucleic acid types (Vitzthum

et al., 1999; Zipper *et al*., 2004). The dsDNA is concentrated within the capsid and accordingly is detectable as dots under 1000x magnification using epifluorescent microscopy (Noble and Fuhrman, 1998). Viral diversity was assessed based on morphological data obtained using Transmission Electron Microscopy (TEM) (Williamson *et al*., 2005).

The search for bacteriophages can be carried out directly in the environment of bacteria or from lysogenic cells (Loessner, 1991). Overlay methods have been used extensively for the isolation of mycobacteriophages from various sources without amplification (Pedulla *et al*., 2003). The principle of the method is the extraction of virus particles from samples using phage buffer, after centrifugation and filtration resulting supernatants are plated directly on solid overlays containing 0.35% agar and *M. smegmatis* mc^2 155, followed by incubation. Phages are identified as individual plaques on lawn of *M. smegmatis* (Pedulla *et al*., 2003; Hatfull *et al.,* 2006).

The primary isolation of the following mycobacteriophages employed *M. smegmatis* as host. Mycobacteriophage D29, a lytic phage that infects both fast and slow-growing mycobacterial species, was isolated from soil by Froman *et al*. (1954). Bxz2 was isolated from environmental samples from the Bronx Zoo by the William R. Jacobs laboratory at the Albert Einstein College of Medicine, New York. Bxb1 is a temperate phage and forms turbid plaques on lawns of *M. smegmatis* from which stable lysogens can be recovered. Mycobacteriophage Bxb1 was isolated by William R. Jacobs from a soil sample after enrichment on a culture of *M. smegmatis* mc^2155 (Mediavilla *et al*., 2000). Mycobacteriophage Tweety was isolated from a soil sample from the Oakland district of Pittsburgh, and was identified (without amplification) as a pfu on a lawn of *M. smegmatis* mc^2 155 (Pham *et al*., 2007).

Morris *et al*. (2008) described a novel mycobacteriophage (Giles), a distinct relative of other phages that infect the same *M. smegmatis* mc^2 155. Mycobacteriophage Giles was identified by direct plating on lawns of *M. smegmatis* using an extract of soil and debris recovered from under a log pile near Pittsburgh, PA, with phage buffer.

Doke (1960) isolated the temperate phage L5 from cultures of *M. smegmatis*. TM4 was isolated from an *M. avium* culture after treatment with mitomycin C (Timme and Brennan, 1984).

3.2. Aims and Objectives

It has been suggested that phages associated with bacteria may make an important contribution to the evolution of bacterial genomes and therefore to the development of bacterial pathogenicity (Boyd and Brussow, 2002). Data from sequenced mycobacteria genomes suggests that there is minimal prophage content; however, it is not known whether this is generally true, or reflects a limited sample. Towards addressing this question we were interested in seeing if we could use standard microbiological approaches to assess the presence of inducible phages associated with our isolated mycobacteria and whether we could isolate mycobacteriophages directly from soil that contained mycobacteria.

3.2.1. Specific objectives

- Isolation of spontaneously inducible prophages
- Isolation of soil-associated mycobacteriophages
- Characterization of isolated phage/s

3.3. Materials and Methods

All oligonucleotides, restriction enzymes, and host and strains used in this study are shown in Table 6.

Table 6. Bacteria strains, bacteriophage and Oligonucleotides used in this study.

Designated Name	Description	Source
Host and Phage Strains:		
M. smegmatis mc^2155	Laboratory strain; fast-growing mycobacteria Prof. V. Mizrahi. NHLS, University of the Witwatersrand	
M. smegmatis mc^2155 L5 lysogen	Wild type strain that carry L5 prophage	Belinda, S., MSc student; University of the Witwatersrand
Mycobacteriophage L5	Well characterised temperate mycobacteriophage that forms lysogens with several mycobacteria including *M. smegmatis* mc^2155.	Marissa Pedulla; Michigan State University, (Hatfull and Sarkis, 1993)
Environmental mycobacteria isolates		Isolated in this study (see previous chapter)
Oligonucleotides:		
L5 Forward primer	5'CTTGGATCCTCCCGCTGCGC; forward primer for the amplification of the gp71 L5 repressor gene. Identical to cI repressor in phage λ (relative to the start codon) of gp71.	This study
L5 Reverse primer	5'AATTCTTGCAGACCCCTGGA; Reverse primer of the gp71 L5 repressor gene.	This study
Restriction enzymes:		
*Eco*RI	GAATTC	Fermentas
*Taq*I	TCGA	Fermentas
*Hin*fI	GANTC	Fermentas
*Bgl*II	AGATCT	Fermentas

[1] Where N = G, A, T or C

3.3.1. Spontaneous prophage induction.

3.3.1.1. Culture conditions

All culturing was as previously described (section 2.3.2) except for a few minor modifications. Mycobacteria were cultured in liquid 7H9 Middlebrook media essentially as described in Chapter 2 (section 2.3.4). In addition to the 0.3% glucose, the media was also supplemented with 1mM $CaCl_2$ and 1mM $MgSO_4$, which is known to enhance phage attachment (Kasman, 2005; Lindberg and Hellerrqvist, 1971). In the presence or absence of 0.06% Tween 80. The cultures were incubated at either at 37^{o}C or at room temperature with gentle agitation (100rpm) in the absence of Tween 80 since Tween 80 may have a detrimental effect on phage attachment as it is a detergent and may alter the surface properties of the bacteria cell and therefore influence the phage infection (Karnik and Gopinathan, 1980). The bacteria cultures which, in the absence of Tween 80 tended to clump and these clumps were dispersed by passing the bacterial suspension through a 25-gauge syringe needle (Rybniker *et al.,* 2006).

3.3.1.2. Preparation of phage filtrates

Following growth of mycobacteria isolates and *M. smegmatis* mc^{2}155 L5 lysogen as described for two to three days, the broth was centrifuged at 13,000×g for 2 min. to remove bacterial debris and the supernatant fluid was filtered through a Millipore membrane filter with pore diameter of 0.2µM. Following this, the phage filtrates were assayed by the conventional soft-agar layer method of Adams (1959) or by the indicator plate method (see following section).

3.3.2. Phage quantification

As previously mentioned the presence of phage was assayed quantitatively by the standard plaque overlay method (Adams, 1959) and indicator plate method (McNerney *et al*., 2004).

3.3.2.1. Plaque overlay method

Plaque overlays were performed using a method from Hatfull *et al*. (2006), potential phage-containing culture filtrates were diluted in phage buffer (1mM Tris; 1mM $MgSO_4$; 6.8mM NaCl and 1mM $CaCl_2$; pH 7.8) and 100µl was mixed with 900µl culture of *M. smegmatis* mc^2155 that had been prepared by overnight incubation as described (section 2.3.4) until an (OD_{600nm} 1) was reached. The phage was allowed to attach to the cells by room temperature incubation for 10 min. This was then mixed with 0.9ml semi-solid agar and poured onto solid agar (7H10 Middlbrook supplemented with 1mM $CaCl_2$, 1mM $MgSO_4$, and 0.3% glucose); incubated at 37^{o}C and monitored daily for plaque development. Plaques were enumerated and plaque forming units (pfu) per ml was determined.

3.3.2.2. Indicator plate

In order to perform indicator plate experiment, a method adapted from McNerney *et al*. (2004) was used. Indicator plates were prepared with Middlebrook 7H10 medium supplemented by 1mM $CaCl_2$; 0.3% glucose; 1mM $MgSO_4$ and 10% liquid culture of *M. smegmatis* mc^2155 grown to an OD_{600nm} of 0.7 to 1.0 in the presence of 0.06% Tween 80 as described previously (section 2.3.4). Culture filtrates prepared as described in the previous section were subjected to a serial dilution in phage buffer and 0.1 ml were plated onto indicator plates and incubated at 37^{o}C. Plates were monitored daily for the development of clearings. Clearings were enumerated and pfu/ml, estimated.

3.3.3. Phage purification

Phages were purified by removing a well isolated plaque from an overlay plate, or clearing from an indicator plate, using tips or a sterile Pasteur pipette and resuspending this in 1 ml of phage buffer. This was then centrifuged at 13 000rpm for 2 min, and 0.1 ml of the supernatant was then plated by standard plaque overlay or on a indicator plate containing sensitive *M. smegmatis* mc^2155 as described (section 3. 3. 2). This process was repeated two or three times.

3.3.4. Isolation of mycobacteriophages directly from soil

Five grams of soil samples were added to Falcon tubes (Lasec) containing 20 ml of phage buffer, followed by gently shaking manually to release phage into the bulk liquid for 20 min. After centrifugation at 6,000×g for 5 min, 10 ml of the supernatant was then collected and filtered using a 0.2μM filter to acquire the phage containing filterable fraction. A volume of 0.1 ml of this was then plated onto mycobacteria-containing indicator plates prepared as previously described (section 3.3.2.2). The plates were incubated for two to seven days at 37^{o}C. Clearings that appeared during incubation were then picked and re-isolated twice as described (section 3.3.3).

3.3.5. Preparation of Large Scale phage

Large scale phage preparation were adapted from Sambrook *et al.* (1989) and Prof. Hatfull**:** **(**http://www.pitt.edu/~gfh/**)**, 30 indicator plates with more than 1000 visible plaques were used. Approximately 4 ml phage buffer was poured onto each indicator plate to cover the surface and placed at 4^{o}C overnight. The phage buffer recovered from each plate was then pooled and centrifuged at 3500×g for 5 min. to remove particulate debris. The supernatant was then transferred to a sterile centrifuge tube to precipitate the phage using 10% PEG 6000 and NaCl adjusted to 1M. This was gently shaken overnight at 4^{o}C and then centrifuged at 3,500×g for 5 min. Ice-cold phage buffer was used to resuspend the pellet by gentle agitation overnight at 4^{o}C.

The resuspended phage preparation was then subjected to cesium chloride density gradient centrifugation. Three different concentrations of cesium chloride (1.45 g/ml, 1.50 g/ml, and 1.70 g/ml) were prepared in phage buffer and transferred to a clear polypropylene ultracentrifuge tube to generate a step gradient with 1.7g/ml at the bottom and 1.45 g/ml contained at the top. Finally the phage, which was resuspended in 0.5 g cesium chloride for every 1ml of phage, was layered at the top of the gradient and centrifuged at 30,000 rpm for 2h at 4°C in a Beckman Ultracentrifuge L7-55, using a rotor with serial number 782. A bluish band, representing the phage material, was collected by using a sterile Pasteur pipette. This was then dialyzed against 500 ml of cold phage buffer at 4^{o}C overnight. After dialysis, the mycobacteriophage were fractionated and stored at 4°C and -20^{o}C.

3.3.6. Isolation of phage DNA

Phage DNA isolation method used in this study was from Sambrook *et al.* (1989) and Prof. Hatfull: **(**http://www.pitt.edu/~gfh/**),** cesium chloride purified phage (approximately 10^9 pfu/ml) was mixed with an equal volume of Tris (pH 8) buffer-equilibrated phenol and mixed by gentle inversion. This was then centrifuged at 13,000 rpm in a bench top centrifuge for 5 min. to separate the phases. The upper, aqueous layer was carefully transferred to a new tube and then chloroform extracted by mixing the aqueous phase with an equal volume of water-saturated chloroform and gently mixed. This was then centrifuged as described previously to separate the phases. The chloroform extraction was then repeated and DNA was concentrated by ethanol precipitation, by mixing with 2.5 volumes of 95% ethanol and a tenth the volume of 3 M sodium acetate (pH 5.6) and placed on dry ice for at least 10 min. or by incubation at -20^{o}C overnight. To recover the precipitated DNA, the tube was centrifuged at 14, 000 rpm at 4^{o}C for 30 min. in a benchtop centrifuge. The supernatant was then discarded and the DNA pellet, rinsed with ethanol (70%). After a second centrifugation, the supernatant again was discarded and the DNA pellet was air dried and re-dissolved in TE buffer (Per 100ml dH_20: 0.121g Tris-Cl and 0.0372g EDTA, pH 8) at room temperature.

3.3.7. Restriction analysis and estimation of size of restricted fragnments of digested phage genomic DNA

The phage DNA digests were set up according to Campbell *et al.* (2002), using approximately 1µg of the isolated DNA (determined by visual comparison of DNA run in parallel to a DNA standard of known concentration on an agarose gel) and essentially according to supplier's specifications. The DNA was incubated at 37^{o}C overnight. The digested phage DNA was run on a 1% TAE gel as described above (section 2.3.5.3). Size of digested A22 DNA were determined by constructing a semi-logarithmic standard of marker DNA using a 1Kb ladder (Fermentas) and then reading off the digested phage DNA using migration distance of each band in the size marker against its size in base pairs. We were unable to get good digested L5 DNA so to calculate the different sizes of restricted DNA, the complete genome of L5 was downloaded from NCBI [accession number: Z18946]. This complete genome was analyzed using DNAMAN software package version 4.15 (Lynnon Biosoft), to calculate the sizes of restriction products for L5.

3.3.8. Epifluorescence Microscopy (EFM) to detect free phage particles

The method was adapted from Noble and Fuhrman (1998); Naicker and Durbach (2007), environmental mycobacteria isolates, L5 lysogen and wild type *M. smegmatis* mc^2155 isolates phage filtrates were prepared by picking colonies, that were freshly grown in Middlebrook 7H10 agar plates as described in Chapter 2 (section 2.3.4), with a sterile inoculation loop and suspending these into 1ml phage buffer. Each of the bacterial suspensions was mixed by repeat pipetting to disperse the colony. The bacterial cells were centrifuged at 13,000rpm for 5 min. in a benchtop centrifuge followed by filtration through disposable 0.2µM filter to remove cells and debris. The suspensions were then each filtered separately onto a 0.02µm pore size Al_2O_3 Anodisc 25 membrane filter (Whatman, Kent, UK) 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 and stained with SYBR Green 1 as described below.

SYBR Green 1, which is able to penetrate the phage capsid and intercalates with its nucleic acid (with a strong preference for dsDNA) 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 filtered through a 0.2µM filter 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).

The filtered material was then stained by submerging the Anodisc filter in 0.25% SYBR Green 1 stain working solution with sample-side facing up for 15 min. in the dark. After the staining period, the filter was picked up and any remaining moisture was then carefully wiped off by touching the back side of the membrane with Kimwipe (Kimberly-Clark). The Anodisc filter was then mounted sample-side up, on a glass slide. A few drops of mounting solution (50% Phosphate buffer saline (PBS, 0.05M Na_2HPO4, 0.85% NaCl, pH 7.5), 50% glycerol, and 0.1% 2-phenylenediamine (Sigma Chemicals; made freshly from a freshly made 10% aqueous stock solution)

was added onto the Anodisc filter and covered with a 25-mm square cover slip. The slides were observed under a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Poughkeepsie, NY, USA), under blue excitation at 1000× magnification. Two to three fields per sample were digitally photographed using the Axiocam mounted digital camera.

3.3.9. PCR amplification of phage DNA using L5 gp71 specific primers.

In order to distinguish the new phage isolates from mycobacteriophage L5, a L5 specific PCR amplification that targets gene 71 of L5 mycobacteriophage was used. For the PCR amplification, a single plaque picked from an overlay or indicator plate was resuspended in 1 ml phage buffer. The DNA was extracted by boiling the sample for 10 min. This was then centrifuged at 13,000 rpm in a benchtop centrifuge for 10 min. and the supernatants were subsequently used for PCR amplification. The primers used are shown in Table 6. The PCR was performed as described above in (section 2.3.5.2), with 55^{o}C as annealing temperature. The PCR products were analyzed on 1% Agarose gels described in (section 2.3.5.3).

3.3.10. Transmission electron microscopy (TEM).

Transmission electron microscopy of the mycobacteriophages was carried out in order to observe the morphology of these phages as described by (Wommack *et al.,* 2000). A suspension of CsCl-purified phage (titer = 10^{9} pfu/ml) was applied to a carbon coated formvar grids for 20 min. The excess fluid was gently drawn off with filter paper. Five microliters of stain (1% uranyl acetate in 45% ethanol) was then placed on the grid for 3 min. and then the excess fluid was drawn off with filter paper. The grids were washed with double deionized water, and the excess fluid, again, drawn off. The grids were then dried at room temperature before being viewed under 50,000 to 100,000 magnification using a transmission electron microscope (JOEL-JEM-100S, Japan) TEM (Naicker and Durbach, 2007).

3.4. Results

3.4.1. Isolation of mycobacteria-associated prophages

In this study we tested the supernatants of the mycobacteria isolates without prior exposure to physical or chemical treatment in order to see if there were prophages associated with the mycobacteria isolates. Since, at least some of the strains were genetically similar to the laboratory strain *M. smegmatis* mc^2155, we expected that if they contained prophage they should be able to plaque on *M. smegmatis* mc^2155. Several studies have also shown that some mycobacteriophages have wide host ranges (Timme and Brennan, 1984). The data (not shown) obtained demonstrated clearly that we were unable to detect spontaneously inducible phage in the form of plaques using standard overlays, or clearings on indicator plates, with *M. smegmatis* mc^2155 as the sensitive strain, even though phage could be isolated (10^2 pfu/ml) from the *M. smegmatis* mc^2155 L5 lysogen control, using this approach, during log phase. The exclusion of tween, which may affect mycobacteriophage viability (Gadagkar and Gopinathan, 1978), did not affect this result. Extending the sampling time to over 1 week and doing cultures at room temperature also did not affect phage yield.

3.4.2. Epifluorescence Microscopy (EFM) analysis

The results presented in (section 3.4.1). Demonstrated that the standard plaque assay methods for the isolation of spontaneously inducible phages was not successful to detect the presence of phages associated with mycobacteria isolates. This may be explained by the fact that there may not have been prophages, or that the laboratory strain of *M. smegmatis* mc^2155 is insensitive to any spontaneously induced phages. We therefore attempted to identify if there were any phages in the culture filtrates of the environmental mycobacteria 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. A single colony of L5-*M. smegmatis* mc^2155 lysogen was suspended in phage buffer and the filtered supernatant was stained on an Anodisc membrane with SYBR Green I and

tested for the presence of any spontaneously induced L5 phage particles as described previously (section 3.3.1). A *M. smegmatis* mc^2155 wild type cells were also prepared for staining, to serve as a negative control. As can be seen from Fig. 3, the presence of free phage particles in L5 were observed in a single field by EFM as approximately ~110 spots. Since the titre of spontaneously induced prophage from the lysogen is 10^2 pfu/ml it suggests that we may be able to detect as little as 1pfu/ml (Fig. 3D.). Importantly spots were not seen associated with the negative control (Fig. 3C). Similar spots were also not observed with any of the mycobacteria isolates tested in this study, of which two are shown (Fig. 3A and B). All the other isolates essentially looked like these representative ones as well.

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 mycobacteria isolates were prepared, stained with SYBR Green 1, and viewed under EFM as described in the materials and methods (section 3.3.8). A *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.

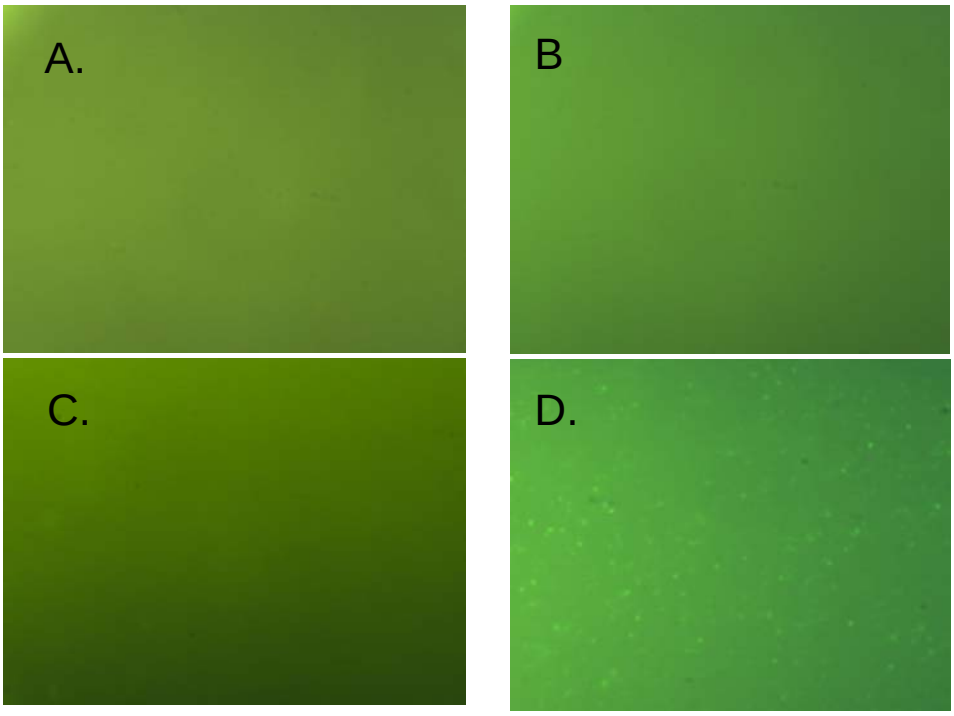


Figure 3. **Epifluorescence Microscopy of mycobacteria colony filtrates**. **A**. mycobacteria isolate S3; **B**. Mycobacteria isolate S9, **C**. *M. smegmatis* mc^2155, **D**. *M. smegmatis* mc^2155 L5 lysogen.

It is clear that EFM is a feasible microscopic technique for screening for the presence of spontaneously induced mycobacteriophages 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 an individual phage in a single field. EFM can be used for direct screening and enumeration of phage particles in solid lysogenic cultures, but failed to detect spontaneously induced mycobacteria-associated prophages, which suggests that these mycobacteria did not contain spontaneously inducible prophages.

3.4.3. Phage isolation

Since we were unable to isolate associated prophages we attempted to isolate free phages directly from soil samples originated from Mpumalanga rural areas, where mycobacteria isolates have been isolated from, to see whether we can find any phages in these environments using the indicator plate or standard plaque overlays which were described previously (section 3.3.2). A *M. smegmatis* mc^2155 wild type cells were used as the sensitive host strain. Using this approach a single plaque was

observed on an indicator plate. This plaque was passaged three times as described (section 3.3.3) to ensure that it was pure. The phage was designated A22.

3.4.4. Host range of A22 and L5 mycobacteriophages in *M. smegmatis* and other mycobacteria isolates.

In order to preliminarily characterise this newly isolated phage we determined the host range of the isolated A22 using the indicator plate method on our previously isolated mycobacteria. A large scale preparation of the phages were prepared and 5µl of this was spotted onto an indicator plate. Plates were incubated for two to seven days and checked regularly for the development of clearings. The results are shown in Table 6, and the data confirmed used standard overlays (not shown).

Table 7. **Host range of A22 in comparison to L5.**

Isolate	Clearing	
	L5	**A22**
M. smegmatis mc^{2}155	+	+
S2[1]	+	+
SB[1]	+	+
S8[1]	+	+
S7[1]	+	+
S1[2]	+	+
S3[2]	-	-
B1[3]	-	-
B2[3]	-	-
B3[3]	-	-
S9[4]	-	-

[1] *M. smegmatis*-like; [2] *M. fortuitum*-like; [3] *M. mucogenicum*-like; [4] *M. aubagnense*-like, according to *hsp65* sequence data (see Chapter 2); +, clearings observed; -, no clearings observed.

It clear that the host range obtained for A22 mycobacteriophage is identical to that of L5 mycobacteriophage. As expected L5 was able to form clearings on *M. smegmatis* mc^{2}155, but was also able to clear the other *M. smegmatis*-like mycobacteria (S2, SB, S7 and S8). The more distantly related *M. mucogenicum*-like mycobacteria (B1, B2, and B3) and *M. aubagnense*-like (S9) could not be cleared. The genetic distance data therefore agreed reasonably well with the plaquing patterns for both phages. Surprisingly both L5 and A22 formed plaques on the *M. fortuitum*-like S1, but not S3. Since A22 and L5 shared a similar host range, it was possible that they were the same or very similar phages.

3.4.5. Differentiation of A22 and L5 phage by infecting *M. smegmatis* mc^{2}155 L5 lysogen.

We reasoned that if A22 phage differed from L5 phage it may be able to infect a *M. smegmatis* mc^{2}155 L5 lysogen whereas L5 would be incapable of doing so due to superinfection immunity (Donnelly-Wu *et al*., 1993). As can be seen from Fig. 4, phage A22, isolated from soil samples, was able to produce clearings on a *M. smegmatis* mc^{2}155 L5 lysogen (Fig. 4A), whereas L5, as expected, failed to do so (Fig. 4D), suggesting that phage A22 is distinct from L5. Both phages were, as has been previously demonstrated, able to produce clearings on *M. smegmatis* mc^{2}155 indicator plates.

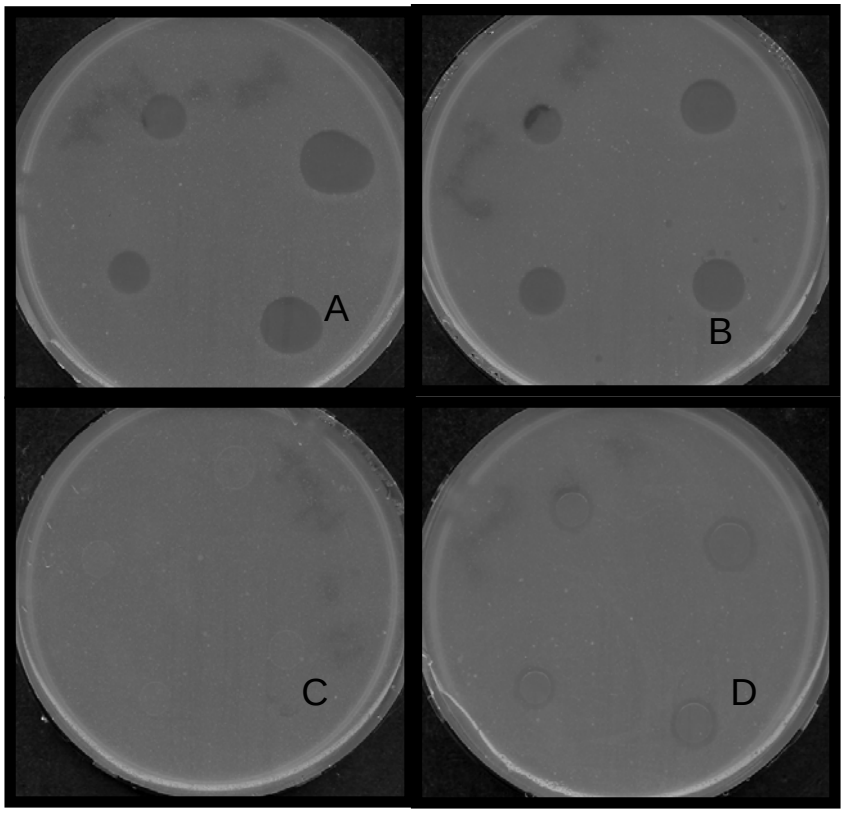


Figure 4. **A22 and L5 on *M. smegmatis* mc^{2}155 and L5 lysogen derivative**. **A**. A22 on *M. smegmatis* mc^{2}155 L5 Lysogen; **B.** A22 on *M. smegmatis* mc^{2}155; **C**. L5 on *M. smegmatis* mc^{2}155 L5 Lysogen; and **D**. L5 on *M. smegmatis* mc^{2}155.

3.4.6. PCR amplification of gp71 gene in A22 and L5 phage using L5gp71 specific primers.

Given that A22 appears to be able to infect a L5 lysogenic derivative of *M. smegmatis* mc^2155, we decided to confirm this result by seeing whether we were able to amplify, by PCR, the L5-specific repressor gene in A22. Amplification of L5gp71 of the anticipated size was obtained for L5 as can be seen in Fig. 5, lane 2. However A22 phage failed to yield an amplification product (Fig. 5, lane 3) and yielded an identical result to the no template control (Fig. 5, lane 1). This lent additional evidence that A22 differed from the well characterized L5 mycobacteriophage.

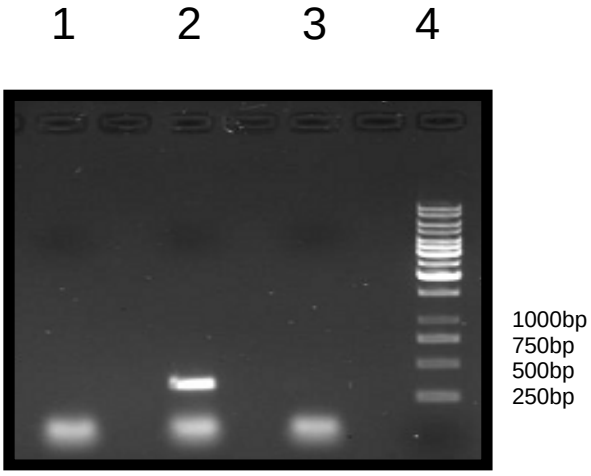


Figure 5. Agarose gel electrophoresis of L5gp71-specific PCR. Lane 1, no template included; lane 2 L5 DNA; lane 3, A22 DNA; lane 4 DNA size marker (DNA ladder 1kb, Fermentas).

3.4.7. Transmission electron microscopy (TEM) comparison of A22 and L5 phage morphology

Since our initial data suggested that phage A22 and L5 were different, we next looked at virion morphology to see if there were detectable morphological differences. The mycobacteriophage were identified by using morphological criteria outlined by the International Committee on Taxonomy of Viruses (ICTVdB, 2006). Morphological study revealed that, A22 phage has a morphology typical of the Siphoviridae, with an isometric head that had 60nm in diameter and a non-contractile tail that has 150 nm

long (Fig. 6a). This morphology is similar to the virion structure of the L5 phages of the Siphoviridae family, which have characteristic isometric heads and long, flexible, non-contractile tails. This morphology is the most common one found among mycobacteriophages (Hatfull *et al.,* 2006).

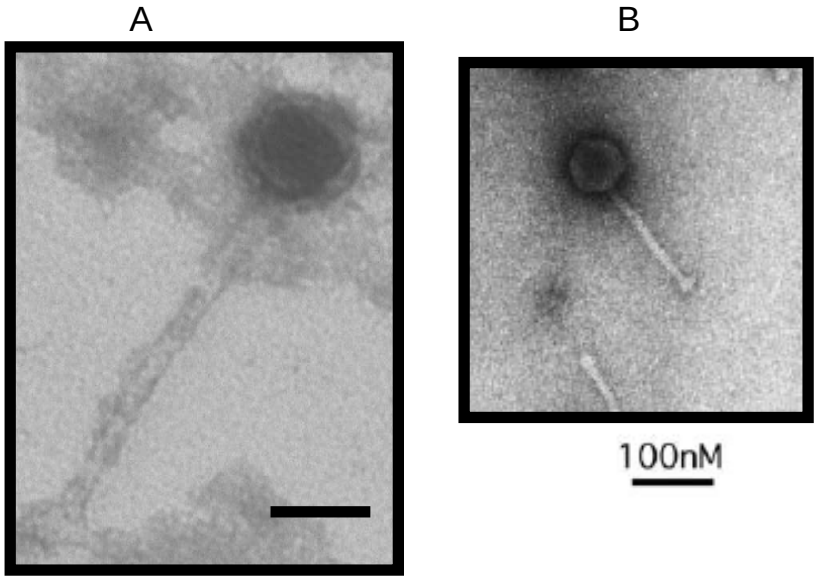


Figure 6. Transmission Electron Micrographs of A22 viron. **A**. A22 (bar represents 100.000x); **B**. L5 image, together with scale bar, taken from Pedulla *et al*. (2003).

3.4.8. Restriction digests of A22 DNA

Since many phages are similar in morphology, it was necessary to develop some studies to estimate the difference of our novel A22 phage isolate. DNA restriction pattern can differ radically between phages of even similar morphology, and is therefore a better hallmark of difference (Pedulla *et al*., 2003). After digestion of A22 DNA, the resulting DNA fragments were separated by agarose gel electrophoresis. As can be seen from Fig. 7 all restriction enzymes digested the DNA, since they all ran differently to the uncut control (Fig. 7, lane 2). Both *Eco*RI (Fig. 7, lane 3) and *Bgl*II (Fig. 7, lane 6) yielded similar size ranges. Since both recognise 6bp target sequences and have the same GC content this was expected. The smallest fragment size range of DNA was recovered from the *Taq*1 digest (Fig. 7, lane 4) and was larger than the *Hin*fI generated size range, which recognizes a 5bp target sequence, even though there is redundancy in the middle base of the sequence (5'GANTC 3'). We were unable to directly compare digested A22 DNA with that of L5 as our L5 DNA

failed to digest (not shown). We therefore calculated the expected fragment sizes of L5 DNA (obtained from Genbank) digested separately with *Bgl*II and *Eco*RI using an algorithm contained in the DNAMAN suite of programs. This is shown in Table 8. We did note that the failure to see bands smaller than 3,000bp with *Eco*RI (lane 3) and *Bgl*II (lane 6) and noted that this may have been due to insufficiently digested DNA. We therefore excluded all sizes less than 3,000bp from our analysis. As can be seen from Table 8 the *Bgl*II digest appears to have no bands in common, but more significantly, whereas our *Eco*RI digest of A22 yielded at least 6 fragments, the L5 genome contains no *Eco*RI sites confirming that L5 is genetically distinct from A22.

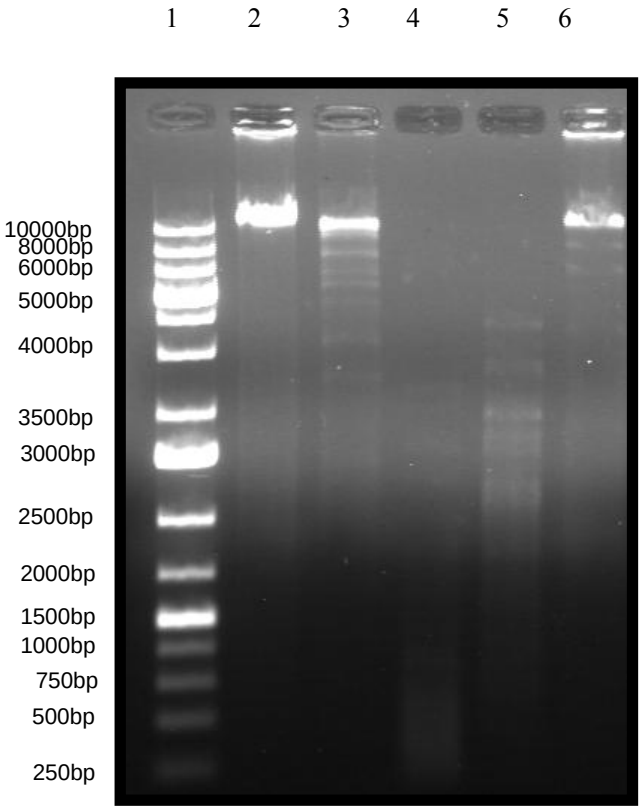


Figure 7. Restriction digest analysis of A22 DNA**;** Lane 1, DNA ladder 1kb, Lane 2, Uncut DNA; Lane 3, *Eco*R1; Lane 4, *Taq*1; Lane 5, *Hin*f1 and Lane 6, *Bg1*11

Table 8. Table of A22 and L5 digest fragment lengths

	***Bg1*11/Fragments (bp)**	***Eco*R1/Fragments (bp)**
A22 phage	9951, 7840 5800	3,300; 3,891; 4,910; 5,850; 7,950; 9984
L5 phage	3,350; 4,612; 4802; 10,993; 14,336	no sites

3.5. Discussion and conclusion

Two approaches have been used to study spontaneous induction of temperate phages and the presence of prophage in ten mycobacteria isolates isolated from soil samples. Previous research reports have demonstrated that UV light is a reliable method for the induction of prophages (Lwoff *et al.,* 1950). Recently mitomycin C has been found to be a better inducing agent of prophages (Cochran *et al*., 1998; Jiang and Paul, 1996; Williamson *et al*., 2008). However phages are spontaneously released without any inducing agent due to several stochastic processes related to host bacterial DNA replication (Joklik *et al*., 1980; Kilic *et al.,* 2001). Based on this observation, the present study has focused on the spontaneous induction of temperate phage without prior exposure to physical or chemical agents from environmental mycobacteria isolated from soil samples. A *M. smegmatis* mc^2155 and it L5 lysogenic derivative were included as positive and negative control. According to our findings no inducible phage could be detected. This result suggests that among our mycobacteria isolates there may not have been inducible phages or the phage induced may have been insensitive to *M. smegmatis* mc^2155. The levels of spontaneously generated phage may have also been low (Czyz *et al*., 2001). However the failure of EFM, which can detect as little as 1pfu/ml, does suggest a likely absence of inducible phage.

In order to determine the presence of bacteriophages in mycobacteria isolates, after spontaneous induction method, another approach based on a fluorescent staining method using epifluorescence microscopy was used (Hara *et al*., 1999). More recently EFM has been shown to result in more accurate and more easily performed enumeration of viruses (Weinbauer and Suttle, 1997). Based in these observations, we decided to use this method whether its can provide complementary information, such as the detection of mycobacteriophages that are probably attached to or internalized into host cells or to differentiate different morphologies. The data shown in Fig. 3, demonstrated that for over all mycobacteria isolates no phage has been detected using this method, except the positive control *M. smegmatis* L5 lysogen that has been included to this experiment, the stained viruses are visible as small green fluorescent spots with epifluorescence microscopy (Fig. 3). This observation suggested that, the mycobacteria isolates were not carrying any inducible bacteriophages.

Since no temperate phages were obtained from environmental mycobacteria isolates, strategies were shifted to isolate mycobacteriophages directly from soil samples where the mycobacteria isolates were isolated.

We have isolated and characterized a novel phage designed A22 phage from soil samples collected in rural areas in Mpumalanga, South Africa. This phage isolate contains many features that are consistent with the L5 mycobacteriophage; however, based upon PCR amplification of gp71 gene using L5 gp71 specific primers analysis (Fig. 6), the absence of amplification, and infection of the L5 lysogen see (Fig. 5), we believe this to be a new mycobacteriophage. In this study, we describe the results of the differences found between this new isolate and the well characterized L5 phage using Transmission electron microscopy and restriction analysis. Host range analyses were also performed. Over the past few years many reports have demonstrated that phages are likely to be numerically the most prominent biological systems on earth and only a few phage groups have been intensively studied by sequencing such as coliphages, mycobacteriophages, and dairy phages (Sandra *et al*., 2004).

The phenotypic difference between L5 and the isolate A22 were examined by electron microscopy. The phage was identified by using morphological criteria outlined by the International committee on Taxonomy of Viruses (ICTVdB, 2006). Morphological studies revealed that A22 mycobacteriophage appeared to have a non-contractile tail of approximately 150 nm with an isometric head of approximately 60 nm. The phage could be assigned to the family *Siphoviridae.* The morphology of A22 particles appeared to be similar to that of L5, D29 (Ford *et al*., 1998), Bxb1 (Mediavilla *et al.,* 2000) and Tweety phage (Pham *et al*., 2007). According to these criteria, all of the phages (A22 and L5) belong to the order *Caudovirales* (tailed bacteriophages). This morphology is the most common one found among mycobacteriophages (Hatfull *et al*., 2006).

A method similar to the one described by Rybniker *et al*. (2006), for phage typing was used to determine the host range of mycobacteriophage A22 compared to the well known L5 phage. Mycobacteriophage L5 is the best characterized of the mycobacteriophages (Hatfull, 1994). L5 is a temperate phage isolated from a strain of *M. smegmatis* by Doke (1960) that forms stable lysogens in which the phage genome

is integrated into the bacterial attachment site, *attB* (Snapper *et al.,* 1988; Lee *et al.,* 1991). L5 also infects slow-growing mycobacteria such as *M. bovis* bacille Calmette-Guérin (BCG), although rather specific conditions are required for efficient infection (Fullner and Hatfull, 1997). Phage A22 was isolated directly from soil and has a similar host range with L5 phage at least with the fast-growing mycobacteria isolates tested in this study. It is difficult to conclude from our data that A22 phage has the same host range in both slow and fast growing mycobacteria with L5 phage because slow-growing mycobacteria were not tested in this study. Since other phages have restricted host range such as Bxb1 and I3, (which infect only fast-growing mycobacteria) and many phages (e.g. L5, D29 and TM4) infect both fast and slow-growing mycobacterial strains (Mediavilla *et al*., 2000) it would be interesting to determine whether A22 is capable of infecting slow-growing mycobacteria. Furthermore, previous studies of D29 demonstrated that it may be a member of L5-like family of mycobacteriophages. Interestingly, although a virulent phage, D29 is subject to superinfection immunity by L5. In other words it is incapable of infecting an L5 lysogen of *M. smegmatis* mc^2 155 (Ford *et al.,* 1998) which was not the case with A22, which can infect a *M. smegmatis* $mc^2$155 L5 lysogen. This observation revealed that A22 phage may be also different from D29 phage.

The host ranges of the mycobacteriophages are likely to be determined mainly by the structural features at the tips of the tails which are presumed to come into direct contact with the bacterial cell surface (Mediavilla *et al*., 2000). The minor tail proteins of L5 are encoded by gp27 and gp28 and are the most likely candidates for forming these structures (Mediavilla *et al*., 2000). As A22 appears to have similar host range, it is reasonable to suppose that there may be sequence similarity in the minor tail proteins

L5 is a temperate phage and forms turbid plaques on lawns of *M. smegmatis* from which stable lysogens can be recovered. These lysogens are susceptible to A22 infection, this susceptibility to infection has been reported with Bxb1 and TM4 phages (Mediavilla *et al.,* 2000), but are immune to superinfection by L5 (Fig. 4). This immunity can also be observed with D29 phage (Mediavilla *et al*., 2000). This data suggests either that A22 is a virulent phage, or like Bxb1 and TM4, its mechanism of repression is different. This remains to be shown.

Although the number of mycobacteriophage genomes sequenced to date is still small, L5, D29 and Bxb1 have been demonstrated to share similar genomic architectures (in spite of their diverse geographical origins), form a small group and are more similar to each other than they are to TM4 (Mediavilla *et al*., 2000).

Restriction enzyme analysis was carried out to characterize phage diversity. The result was not robust and does not allow us to distinguish correctly mycobacteriophage A22 from the best characterized mycobacteriophage L5, this can be attributale to the lack of digestible L5 phage genomic DNA. Agarose gel electrophoresis of A22 phage DNA fragments generated with *Eco*R1, *Taq*1, *Hin*f1, and *Bg1*11 are displayed in Fig. 7.

Based on *Eco*R1 and *Bg1*11 restriction digests we estimated the fragment lengths of A22 phage in comparison with L5 phage fragments sites obtained from GenBank (Table 8). Which suggested that A22 phage probably might be different genetically from L5 phage due to the absence of *Eco*R1 sites in the L5 genome. This finding is supported by previously reported comparison of 30 mycobacteriophages which suggested considerable overall diversity at the nucleotide level (Hatfull *et al*., 2006). Although, A22 phage genome has not been completely sequenced, based on our finding, A22 phage sheds considerable new light on phage diversity and evolution.

Further characterization of A22 phage may provide an insight into the diversity of the mycobacteriophage, and may provide further evidence on its identity among other mycobacteriophages such as D29 (Froman *et al*., 1954), Bxb1 (Mediavilla *et al*., 2000), and TM4 (Timme and Brennan, 1984).

CHAPTER 4 - CONCLUDING REMARKS

The key objectives of this thesis were achieved, namely to investigate environmental mycobacteria and associated phages. Although prophage induction was not successful, the data presented in this thesis indicate that mycobacteria and associated viruses are diverse and can be found in every environments as previously reported by numerous workers.

Fifteen mycobacteria isolates were isolated from a variety of sources including, soil, water and drinking water biofilms. Mycobacteria were identified as a colony in Middlebrook 7H10 medium supplemented as described above, purified and characterized further. After PCR amplification of *hsp65* gene using specific pimers *hsp*-for and *hsp*-rev, and their sequences. The isolates presented some percentages of sequence similarities closer to well characterize *Mycobacterium sp* in the Genbank. Spontaneous induction of temperate phages has also been performed, but no inducible temperate phage has been detected, due to this observation, A22 phage has been isolated directly from soil samples using *M. smegmatis* wild type as a host. Phage A22 was identified as individual plaques on lawns of *M. smegmatis*, purified, and characterized. The plaque morphology of this phage was similar to the one of L5 phage. This phage was characterized by electron microscopy and viral morphology compared with L5 phage (Fig. 6). Phage A22 has similar morphology to the L5 phage, with isometric head and long flexible tails.

This study has provided an excellent opportunity to isolate and identify some of environmental mycobacteria and mycobacteriophage directly from environmental samples. The study has covered a variety of techniques used in research, including PCR amplifications, sequencing, Restriction digests and other methods.

The study has shown not only the presence of environmental mycobacteria and phages in environmental samples, but the immense opportunities that lie ahead for the potential application of the tools emerging from such basic research. Studies on environmental mycobacteria and associated phages have very important potential applications in relation to the implication of these microorganisms in human and animal health, especially in immunodeficiency populations. Understanding of the

relationship between environmental mycobacteria and their associated phages may also have significant relevance to treatment strategies with future anti-tuberculosis drugs.

Because this study focused on a selected geographic areas in South Africa. The geographic diversity of our samples were collected in rural areas where the animals live and all those samples were obtained from University of Johannesburg, Mycotoxin Research Laboratory. Consequently, samples have been collected from Mpumalanga, Limpopo and Johannesbug. Within the bounds of these limitations, however, this study suggests that environmental mycobacteria are organized into large, genetically differentiated strains. Although this study does not provide direct evidence for the isolation of temperate mycobacteriophages from environmental mycobacteria isolates isolated in this study, at least we have isolated mycobacteriophages designated A22 directly from soil samples and this phages was able to infect *M. smegmatis* L5 lysogen where the L5 phage failed to so, due to lack of PCR amplification using gp71 specific primers, we believe that this new isolate phage A22 is genetically different from the well characterized L5 phage. Although, it has been difficult to confirm when we performed restriction enzyme analysis using *Eco*RI, *Taq*I, *BaI*II, and *Hin*fI to see whether our A22 phage isolate could have similar recognition sites with well characterized L5 phage, at least with the result presented in Table 8, suggested that A22 phage might be genetically different from L5. We can not exclude the possibility that A22 phage may have many genetic features in common with others that have been characterized genomically. Further genomic characterization of A22 phage would be beneficial in other to establish clear relationship with well known mycobacteriophages. Testing soil samples to identify previously unknown viruses and to characterize the newly discovered mycobacteriophages for properties and genes could be beneficial to the medical world in the fight against infectious diseases.

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APPENDIX A
Source of Chemicals and Suppliers

7H10 Middlebrook Agar	Cat No 271310	Difco
7H9 Middlebrook Broth	Cat No 262710	Difco
Bio Taq™ DNA Polymerase (5u/µl)	Cat No BIO-2140	Bioline Ltd
Hyperladder 1,		Bioline
Oligo Synthesis	Inqaba Biotechnical Industries (Pty) Ltd	
O'GeneRuler™, 1kb DNA Ladder		Fermentas Inc.
PCR Grade Water: Sabax Injection Water	Cat No	Adcock Ingam
Phenol, TE Equilibrated, pH	Cat No 75829	USB
Restriction Enzymes:		
BgIII		
EcoR1		Fermentas Inc.
HinfI		
TaqI		

APPENDIX B
General Media and Buffers

0.5M EDTA (pH 8)

Per 100ml dH_2O:

18.61g EDTA

Add 90ml of the dH_2O and then adjust to pH 8 using concentrated NaOH. Make the volume up to 100ml, filter sterilize through a 0.2µm syringe filter.

Indicator Plates

Per litre of indicator plate agar:

19g Middlebrook 7H10 agar
5ml Glycerol
900ml dH_2O

Autoclave, 10 min. Allow to cool then add filter sterilized glucose/NaCl stock to a final concentration of 0.2% and 0.085% respectively. Add 100ml of a fresh *M. smegmatis* mc^2 155 culture (OD_{600nm}0.8), mix well.

Luria Broth and Agar

Per litre of dH_2O:

10g Tryptone Powder
5g Yeast Extract
5g NaCl

For Agar, 10g Bacteriological Agar. Autoclave 15-20 min.

Middlbrook 7H9 Broth

Per litre of dH_2O:

4.7g Middlebrook 7H9 powder
2ml Glycerol

Autoclave, 10 min. Allow to cool then add filter sterilized glucose/NaCl stock to a final concentration of 0.2% and 0.085% respectively.

Middlbrook 7H10 Agar

Per litre of dH_2O:

19g Middlebrook 7H10 powder

5ml Glycerol

Autoclave, 10 minutes. Allow to cool then add filter sterilized glucose/NaCl stock to a final concentration of 0.2% and 0.085% respectively.

Phage Buffer

Prepare at least one litre, containing:

10mM Tris (pH 8)

10mM $MgSO_4$

68mM NaCl

Adjust the pH to 7.8, then autoclave to sterilize. When cool, add $CaCl_2$ that has been filter sterilized through a 0.2µm filter to a final concentration of 10mM.

Soft Agar for Overlays

Prepare Luria Agar with 7g Bacteriological Agar per litre. Autoclave 15-20 min.

10x TAE Buffer

Per litre of dH_2O:

48.4g Tris Base

11.42ml Glacial Acetic Acid

20ml 0.5M EDTA (pH 8)

Make up to 1 litre. Autoclave to sterilize.

APPENDIX C
Growth curve analysis of environmental mycobacteria

