Investigating the *in vitro* roles played by the major adhesins HBHA and MTP in the pathogenesis of *M. tuberculosis*, in a novel double gene knock-out mutant strain.

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Submitted in fulfilment of the academic requirements for the degree of
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As the candidate's supervisor I have approved this thesis/dissertation for submission.

Signature:_____ Name: Prof. Manormoney Pillay Date: _____

Preface

The experimental work described in this thesis was carried out in the School of Laboratory Medicine & Medical Sciences, Department of Medical Microbiology, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, from January 2013 to May 2018, under the supervision of Prof. Manormoney Pillay.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

Declaration 1: Plagiarism

I, Viveshree Shalom Govender, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research. It is being submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in Medicine (Medical Microbiology) at the University of KwaZulu-Natal.

2. This thesis has not been submitted for any degree or examination at this or any other university.

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Declaration 2: Publications

The publications that constitute this thesis (in print, in press and submitted), as well as the contribution that my co-authors and I have made to each of the manuscripts, are presented herein.

Publication 1:

Govender, V.S., Ramsugit, S. and Pillay, M. 2014. *Mycobacterium tuberculosis* adhesins: potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets. Microbiology. 160 (pg.1821-1831).

Author contributions

Prof. M. Pillay, Dr. S. Ramsugit and I conceptualized and planned the review. Dr Ramsugit and Ms V.S. Govender are joint first authors. Dr. Ramsugit and I conducted all literature searches, wrote the paper and compiled the reference list. Prof. Pillay provided invaluable input and reviewed all manuscript drafts, with critical commentary.

Manuscript 2:

Govender, V.S., Jain, P., Larsen, M.H. and Pillay, M. *Mycobacterium tuberculosis* heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) are essential for *in vitro* growth, but not viability and biofilm production.

To be submitted to: ***"Microbiology"***

Author contributions

I conceptualized and designed the study, conducted all the experimental work, processed and analyzed the data and wrote the manuscript. Dr. Jain and Prof. Larsen provided the phages and their genetic information, as well as technical expertise. Prof. Larsen carried out additional WGS confirmation of all strains. Prof. M. Pillay, Dr. Jain and Prof. Larsen guided the study design, provided extensive support during technical troubleshooting of experimental work. Prof. M. Pillay reviewed and edited all the drafts of the manuscripts, with supporting critical assessment.

Manuscript 3:

Govender, V.S. and Pillay, M. "*Mycobacterium tuberculosis* heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) proteins facilitate adhesion to and invasion of A549 pulmonary epithelial cells."

To be submitted to: ***"Tuberculosis"***

Author contributions

I conceptualized and designed the study, conducted all the experimental work, processed and analyzed the data and wrote the manuscript. Prof M. Pillay guided the study design, provided extensive support during technical troubleshooting of experimental work. Prof. M. Pillay reviewed and edited all the drafts of the manuscripts, with supporting critical assessment.

Signed: ..... Date:.....

Viveshree Shalom Govender

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Prof. Manormoney Pillay

Conference presentations emanating from this thesis

College of Health Sciences Research Symposium 2017
Durban, South Africa
5-6 October, 2017
Oral presentation:
"Mycobacterium tuberculosis heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) are essential for *in vitro* growth, but not viability and biofilm production."

Federation of Infectious Diseases Societies of Southern Africa Conference 2017
Cape Town, South Africa
9-11 November, 2017
Poster presentation:
"*Mycobacterium tuberculo*sis heparin binding haemagglutinin adhesin (HBHA) & curli pili (MTP) are essential for *in vitro* growth, but not viability & biofilm production."

Dedication

"The best and safest method of philosophizing seems to be, first to enquire diligently into the properties of things, and to establish these properties by experiment, and then to proceed more slowly to hypothesis for the explanation of them. For hypotheses should be employed only in explaining the properties of things, but not assumed in determining them, unless so far as they may furnish experiments."

- Sir Isaac Newton

Unbound curiosity and precise experimentation are both crucial components that drive scientific knowledge, but along different paths. Sometimes, both avenues converge to allow the passionately motivated to gain access to an unimaginable pool of untapped knowledge. I have been blessed to be curious in my precise experimentation, and have been able to dive deeply into the unknown.

This thesis is dedicated to those who have carried me on their shoulders so that I may see further and be granted the privilege of learning, and the honour of gaining true knowledge. When you recognize that success comes from standing on the shoulders of others, it humbles you. I am forever humbled by your unwavering support, strength and belief in me during my journey through academia. Words will never do justice in describing my gratitude for the sacrifices that you have made and the hardships that you have endured in your lifetime, to secure my lifetime.

*To my phenomenal father, Marimutu "Siva" Govender – you are my whole life! I am nothing without **you**. This is a culmination of our combined endeavours over the past 32 years, and I hope it makes your heart happy.*

To those no longer with me - Philip Govender, Lava Govender, Ganes Govender, Vixon Prakash Govender, Billy Govender, Bosamma Pillay, Sal Govender, Anil Govender:

"If I can aspire to half the success of my elders, then I have achieved in life"

Thank you for the selfless sacrifices that you have made for the Govenders – we are all nothing without your shoulders.

To my divine solace,

I am blessed to have your grace – I am grateful for it all, the hardships and the successes. Thank you for lifting me up from the darkness, for bringing me back into the light. For making me shine brighter than ever before, and for showing me all the colours of my rainbow. Aum Namah Shivaya.

Acknowledgements

To my supervisor, Prof. Manormoney Pillay (Dr. P.), thank you for taking a gamble on me and affording me a second chance at a doctorate. I will forever be grateful to you for defying others and placing all your bets on a wildcard with insane odds stacked against her. Our colourful relationship is a true example of opposites in action. Your kindness, patience, tolerance, knowledge and support during the tenure of this project cannot go without mention.

Thank you to the National Research Foundation, and the College of Health Sciences for financial assistance. To the Africa Centre for unconditional 24/7 use of their molecular laboratory, Sanger sequencer, real-rime and conventional PCR analysers – I am sincerely grateful.

To my parents, Siva and Nagie, for 32 years of dedication, love, financial support, patience, tolerance, understanding, faith and care - I hope the completion of this journey will do your sacrifices justice. Thank you to my main moms, my rock, my precious Indrani Ma, for her unwavering moral support, motivation, wit, "big word for the day", scoldings, smile, and pristinely pure love – she has secured an honorary doctorate with me and is the reason I am blessed as *Dvai-mathura*. You are my sanity, the Morty to my Rick. To my late uncle, Ganes "G" Govender, thank you for your sanctuary, love and support – I hope you now have real reason to sing my praises, wherever your final journey takes you – you are loved and appreciated, always. To my late and dearest grandmother, Bosamma Perumal Pillay, thank you for your love, care and the gift of your genes; thank you for passing on your tenacity and determination. I truly miss you more than anything and wish you were here to toast our best moments.

To my brothers, and sister-in-laws: Vishnu Kevin Govender, Memphis Adrian Govender, Brendan Anen Govender, Alistair Govender, Lakshan Juno Govender, Sarita-Devi Buljeeon-Govender and Shabnum Behari-Govender - thank you for the DMC's, comic relief, junk food parties and for always lending me your ears, your cars, and your spare rooms. Having you all on my side helped more than you know, especially when this project followed Murphy's Law 9 times out of 10, and there was no end in sight. To my beautiful babies, Karina Shrestha, Luxan Simba and Archan Memphis – always strive to be thankful, humble, hard-working, happy, kind and compassionate. Never shy away from a challenge - it will bring you the world!

Thank you to Sanisha Muniram, Sheryll Verajoo, Farina Karim, Georgina Nyawo, Suventha Moodley, Charissa Naidoo, Kavitha Naidoo, Sumira Kuvar, Sasha Jadoo and Siva Danaviah for their camaraderie and support. You girls are an inspiration to women in science!

Lastly, to my nearest and dearest, my loved ones – Sandhir Batohi, Nazeera Kasim, Ashmika Ramanund, Yurisha Ramautar and Renee Reddy: I couldn't have done any of this without you by my side throughout my 14-year career in academia. Your steady support, motivation and encouragement has finally got us there! So, please, go ahead and call me Dr. Govender (for 6 months at *least*, as promised!)…the wait is over!

%	Percent
ΔDM	*Δhbha-mtp* double mutant
°C	degrees celcius
A549	human non-small cell adenocarcinoma pulmonary alveolar epithelial cells
AEC	airway epithelial cells
AES	allelic exchange substrates
ANOVA	one-way analysis of variance
Apa	alanine-proline-rich antigen
ARVs	anti-retro virals
BCG	Bacille Calmette–Guérin
bp	base pair
CD4	cluster of differentiation 4 protein
CDC1551	*Mycobacterium tuberculosis* strain Oshkosh or CSU93
CFP	Culture Filtrate Protein
CFU	colony forming units
DMHC	double mutant *hbhA* complement
DMMC	double mutant *mtp* complement
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide
ECM	extracellular matrix
ESAT-6	Early Secreted Antigenic Target-6
FBS	foetal bovine serum foetal bovine serum
FDA	US Food and Drug Administration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBS	group B Streptococcus
H37Ra	*Mycobacterium tuberculosis* strain H37 - avirulent
H37Rv	*Mycobacterium tuberculosis* strain H37 - virulent
HBHA/*hbhA*	heparin-binding haemagglutinin adhesin
HIV	Human Immunodeficiency Virus
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
IFN-γ	Interferon gamma
IL	Interleukin
INH	Isoniazid
kb	Kilobase
kDa	Kilodalton
KZN	KwaZulu-Natal
LAM	Lipoarabinomannan
LAM4	Latin American Mediterranean strain family
LB	Luria Bertani
M.tb	*Mycobacterium tuberculosis*
Mag	Magnification
MDR-TB	multi-drug resistant TB
Mg	Milligram
MGIT	microbial growth indicator tube
mL	millilitre

mRNA	messenger ribonucleic acid
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTP/*mtp*	*M. tuberculosis* pili
NAATs	nucleic acid amplification tests
Ng	Nanogram
NGS	Next-generation sequencing
Nm	Nanometer
OADC	oleic acid-albumin-dextrose-catalase enrichment
OD	optical density
ORF	Open reading frame
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + Tween 20
PCR	polymerase chain reaction
PE	proline-glutamic acid
phAE280	phage Albert Einstein 280
PIM	phosphatidyinositol mannosides
POC	point-of-care
PPE	proline-proline-glutamic acid
qRT-PCR	quantitative real-time polymerase chain reaction
REMA	Resazurin Microtiter Assay
RFP	Rifampin
RPM	revolutions per minute
sacB	*Bacillus subtilis* levansucrase gene
SEM	standard error of the mean
SLID	Second-line injectable drug
SPSS	Statistical Package for the Social Sciences
TACO	tryphtophan aspartate containing coat protein
TAG	Treatment Action Group
TB	Tuberculosis
TBVI	Tuberculosis Vaccine Initiative
THP-1	Tohoku Hospital Pediatrics-1 (human monocytic acute leukemia cell line)
TNF-α	tumor necrosis factor-α
V9124	F15/LAM4/KZN susceptible strain of *Mycobacterium tuberculosis*
WHO	World Health Organization
XDR-TB	extensively drug-resistant TB
µg	Microgram
µL	Microlitre

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Abstract

Background:

Approximately 1.7 billion people globally are infected with *Mycobacterium tuberculosis (M.tb*), the bacterium responsible for tuberculosis. The disease caused by this unassuming, microscopic pathogen has received the ominous title of the leading cause of death globally from a single infectious agent, ahead of both HIV/AIDS and malaria.

Even though a diverse range of drugs are readily available to treat TB, their impact is barely felt in the face of the rapid development and spread of drug-resistant strains. Coupled with factors such as high disease burdens, low income settings, poor infrastructure and lack of treatment management and infection control measures, the impact of the shortage of effective drugs is further exacerbated by extended time to diagnosis, prolonged initiation of treatment, a lack of cheap, rapid diagnostic tools at the point of care and a shortage of effective drugs to treat drug resistant strains. A lack of treatment compliance results in resistance to old and newly discovered drugs, with extended TB treatment regimens necessitating the use of multiple toxic drugs. These therapies often present with severe side-effects and contra-indications, especially so in HIV-positive patients receiving ARVs. In most cases, this scenario results in eventual fatality.

The development of an efficient and effective vaccine strategy to alleviate the impact and spread of TB is an appealing solution to ease the TB burden. An ideal vaccine against TB should aim to be as efficacious against drug sensitive strains, as they are against drug-resistant strains, irrespective of the resistance genotype. A successful approach toward TB vaccine development must accelerate efforts toward screening and discovery of suitable targets.

The adhesin family of proteins is an attractive option to consider for such study and development. Within this arena, the two major *M.tb* adhesins, HBHA and MTP, offer a potential solution for vaccine development strategies. The 28-kDa heparin-binding haemagglutinin adhesin is the most predominant adhesin on the bacterial surface, facilitating dissemination of *M.tb*. The *M.tb* pili protein (unique to *M.tb* complex

organisms) aids in adherence to epithelial cells and stimulates the humoral immune response. These two candidates show promise for further study and development.

A comprehensive understanding of the mechanisms of interaction of these proteins with the host can be garnered from combining molecular, biochemistry, cellular and microbiology techniques. The availability of phage-based gene knock-out methods allows for the mechanisms behind these interactions to be established, offering a novel method to assess the impact of functional gene expression on the phenotypic traits of an organism.

This study aimed to elucidate the dual contribution of two *M.tb* adhesin proteins, HBHA and MTP, to growth, viability, biofilm production and the host–pathogen interactions in the KZN susceptible strain V9124.

Materials & Methods:

A novel dual-target gene knock out mutant (Δ*hbhA-mtp*) was generated in the F15/LAM4/KZN susceptible strain of *M.tb*, V9124. This phage-mediated process (mycobacterial specialized transduction) removed ~80% of the coding regions within the *hbhA* and *mtp* target genes, replacing them with selective screening markers. This resulted in non-functional HBHA and MTP proteins being expressed in a double mutant knock-out strain. The double mutant strain was used to construct independent Δ*hbhA* and -Δ*mtp* complemented strains. These mutant and complemented strains were used to assess the effect of a combined loss of HBHA and MTP function on growth, viability, biofilm formation, adhesion and invasion capacity of pulmonary epithelial cells, *in vitro*. The F15/LAM4/KZN drug-sensitive strain served as the wildtype control. Growth variances were measured by colony forming unit counts and OD_{600nm} readings, whilst viability was established by the resazurin microplate assay. Biofilm formation was assessed by means of crystal violet staining and quantification of cellular biomass. Adhesion and invasion assays were performed by infection of A549 pulmonary epithelial cells.

Results & Discussion:

The findings demonstrate the significance of these two major adhesins in facilitating growth and biomass formation. OD_{600nm} measurements demonstrated significant differences among the strains across the 21-day period ($p < 0.01$). Optical density

measurements at OD_{600nm} indicate a significant ($p<0.05$) difference in the growth of the double mutant relative to the growth of the wildtype strain. Growth was recovered by ~50% when the double mutant was complemented to restore individual genes. Even though not significant, the removal of *hbhA* and *mtp* concurrently caused an 8.82% reduction in biomass relative to the wild type V9124, and suggest that MTP may have a greater effect on biofilm formation than HBHA. The viability assay indicated no significant ($p>0.05$) differences in the viability of the double mutant relative to the wildtype strain over the 21-day period, showing that these adhesins have no effect on the metabolic viability of *M.tb*. Both adhesins were also found to play a significant role in adhesion and invasion. The ability of the Δ*hbhA-mtp* double mutant strain (ΔDM) to adhere to A549 pulmonary epithelial cells was significantly reduced by 81% relative to the wild-type strain V9124 ($p = 0.00$). The invasive ability of the mutant was also reduced by 83% ($p = 0.00$). The outcomes allude to MTP performing a potentially greater role as an invasin than HBHA in the F15/LAM4/KZN susceptible strain V9124, whilst having similar roles as adhesins.

Conclusions:

The study was able to illustrate a potential compounded effect caused by a loss of function of two major adhesins on bacterial growth, and the vital pathogenic importance of the two major *M.tb* adhesins in promoting *in vitro* growth of the F15/LAM4/KZN susceptible strain. Their similar adhesin roles as presented in this study may allude to adhesion capacity being supplemented by upregulation and expression of additional primary and moonlighting adhesin molecules, as potential compensatory mechanisms implemented by *M.tb* in the absence of either one of the major adhesins.

Over all, the outcomes indicate that HBHA and MTP may, in combination, present an attractive option as novel targets for potential vaccine/drug development, or as biomarkers for development within the point of care diagnostic device arena. Further studies looking into the impact of the absence of these genes on signalling pathways and gene regulation networks (both in *M.tb* and the host) would shed more light into the role they play in facilitating bacterial proliferation, and the potential effect that they may have on host immune modulation. They may also serve to indicate potential compensatory genes to be considered as additional novel therapeutic targets for development alongside *hbhA* and *mtp*.

CHAPTER 1: Introduction & Literature Review

1. Introduction

With one of the first documented descriptions being made by French military doctor Jean-Antoine Villemin in 1868, tuberculosis (TB) (derived from the word tubercle, meaning a small lump or nodule) is one of the oldest diseases known to affect mankind (Osoba, 2004; Shahverdi and Khani, 2017). A microbial disease caused by the acid-fast bacillus, *Mycobacterium tuberculosis* (*M.tb*), it is considered the most common fatal disease for adults worldwide (Shahverdi and Khani, 2017), and continues to cause more adult deaths than any other single infectious disease globally in the modern world (Gagneux, 2012). *M.tb* remains one of the most successful known human pathogens, having infected a quarter of the global human population by developing sophisticated and clever mechanisms to circumvent the host's innate and adaptive immune defences.

Exposure to *M.tb* and TB infection can be followed by several outcomes; rapid clearing of the bacilli by activated innate immunity defence mechanisms, or immediate active disease (Coscolla and Gagneux, 2014). Most commonly, a latent infection may persist, and re-activate should the body become immuno-compromised, sometimes decades following the initial exposure (Coscolla and Gagneux, 2014). Approximately a third of the world's population is regarded as latently infected with *M.tb*, and are at risk of developing active TB (Shahverdi and Khani, 2017). Active disease can be suspected if at least one of four suggestive symptoms is reported, including a prolonged fever, a persistent cough of two weeks or more, weight loss and night sweats (Sulis et al., 2016).

Despite the ever-growing knowledge base available on pathogen transmission, diagnosis and treatment, this ancient disease continues to have a tremendous impact on public health, with much research remaining to be done in order to understand the complex intra- and extra-cellular dynamics of this globally debilitating infectious agent (Osoba, 2004; Fogel, 2015).

Globally, the increasing number of new TB infections and fatalities each year is underpinned by a lack of effective diagnostic tests, drug-resistant strains, and a deadly relationship with the Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS). Reducing the incidence of TB is a critical first step to eradicate

the epidemic (Fogel, 2015), but can only be facilitated by the development of improved diagnostics, and antimicrobial therapies and vaccines (Talbot and Raffa, 2015). This has sparked an intensified search for novel gene/protein targets for the development of therapeutic interventions to reduce the high TB infection burdens worldwide, as well as biomarkers to improve rapid diagnostics and patient outcomes (Zhang et al., 2006; Whitworth et al., 2013; Wallis and Peppard, 2015; Goletti et al., 2016). Currently, the microbiology and pathophysiology of TB are well studied and understood. This knowledge, combined with the application of rapidly evolving molecular techniques, and the power of genome sequencing tools have begun to unveil new insights toward infection control and prevention (Talbot and Raffa, 2015). Molecular biology experimental methods combined with *in vivo* animal studies have the potential to accelerate and support a deeper understanding of the complex biochemistry and cellular complexities of this robust and devastating infectious human pathogen.

Understanding infection mechanisms (specifically how bacteria engage with the host cells), as well as identifying the key bacterial molecules that facilitate host adhesion and entry, is crucial in the search for novel biomarker and therapeutic drug targets (Govender et al., 2014; Ramsugit and Pillay, 2016; Squeglia et al., 2017). Combining knowledge of biochemical, cellular and molecular biology features of *M.tb* thus becomes fundamental in gaining an understanding of the pathogen, in order to design effective strategies with which to fight TB (Chang et al., 2007).

Mycobacterium tuberculosis, like most pathogenic bacteria, requires a diverse range of molecules to survive and persist within the host (Lamichhane, 2011). These components are usually synthesized and presented by the host itself, or are obtained from the surrounding extracellular environment (Lamichhane, 2011). As a result of the significant impact that *M.tb* has had on global health, identifying key molecules central to persistence and longevity within the human host is appealing in the search for novel therapeutic targets (Glickman & Jacobs, 2001). In this arena, the generation and study of gene knockout mutants generated by molecular genetic approaches are extremely useful in that they allow for specific target *M.tb* genes and proteins to be studied in greater detail. This in turn facilitates understanding the genetic basis behind the roles that they may play in conferring virulence and persistence of the organism in the host (Bardarov et al., 2002).

Prior to 1996, the ability to carry out site-specific mutagenesis of *M.tb* was not possible, until allelic exchange mutagenesis with linear DNA substrates (in specific genes) was reported (Balasubramanian et al., 1996). The field rapidly took off, with intensified research giving rise to more efficient and less challenging methods with which to genetically manipulate *M.tb*, including stable mutagenesis techniques (transposon mutagenesis and site-directed mutagenesis) and allelic exchange methods (carried out using long and short linear DNA substrates, phagemid systems, counter-selectable markers such as *sacB* and a range of other recombineering systems) (Lamrabet and Drancourt, 2012).

A high degree of genetic novelty within bacteriophages has positioned them to take center-stage in the advancement of bacterial genetic studies. Within the field of mycobacterial genetics, mutagenesis and allelic exchange can now be performed easily and efficiently using either mycobacterial extrachromosomal or plasmid vectors (Lamrabet and Drancourt, 2012). The modification of phages to encode recombineering systems based on mycobacteriophage Che9c proteins facilitating the development of new tools for manipulation of bacterial genomes (Marinelli et al., 2012) has revolutionized the construction of single-and multi-targeted gene-knockouts and point mutations in the slow-growing *M.tb*.

The method of mycobacteriophage mediated specialized transduction effectively enables DNA transfer in mycobacteria, subverting a number of the challenges posed by the application of electroporation methods of genome manipulation, such as bacterial aggregation (clumping) and a high rate of illegitimate non-specific recombination (Aldovini et al., 1993; Kalpana et al., 1991; Bardarov et al., 2002). By utilizing a naturally-occurring infectious agent in the form of a mycobacterial virus, the general limitations to genetic exchanges (such as the thick, mycolic acid-rich cell wall and polysaccharide, protein and lipid "capsule" which both facilitate mycobacterial growth as a dense "pellicle" structure, preventing DNA from being taken up) can be circumvented (Lamrabet and Drancourt, 2012). The high frequency of successful specific allelic exchange establishes the phage-based methods of specialized transduction as a powerful genetic mechanism for engineering targeted marked and unmarked mutant mycobacterial species (Bardarov et al. 2002).

The primary step in TB pathogenesis is regarded as adhesion of the *M.tb* bacterium to the host cell surface. This process is mediated by microbial surface adhesin molecules, which facilitate the invasion of *M.tb* into cells and the subsequent evasion of the immune system (Ramsugit and Pillay, 2016). Typically surface-exposed, bacterial adhesins additionally serve to establish bacterial cell aggregation by enabling cell-to-cell interaction, and eventual formation of dense microbial biofilm "communities" (Barnhart & Chapman, 2006; da Silva Neto et al., 2009). Several studies have demonstrated the importance of the two primary *M.tb* adhesins, the heparin binding haemagglutinin adhesin (HBHA) and the *M.tb* curli pili protein (MTP) independently in pathogenesis, suggesting that they may be extremely suitable biomarkers for further investigation (Pethe et al., 2001; Alteri et al., 2007; Esposito et al., 2012; Ramsugit et al., 2013; Naidoo and Pillay, 2017). This is due largely to their key roles in facilitating extra-pulmonary dissemination and stimulation of the humoral response, respectively.

Almost all antibiotics currently used in the treatment of *M.tb* target bacterial processes involved in cell growth (Aldridge et al., 2012). Variability in growth patterns in mycobacterial populations create very high frequency population diversity (Aldridge et al., 2012). This heterogeneity in growth states in mycobacterial cells is thought to be the underlying cause of differential antibiotic susceptibility (Aldridge et al., 2012). The quantification of liquid *M.tb* cultures over time to establish growth kinetics is challenged by the bacteria's propensity to form clumps (Lambrecht et al., 1988). The quantification of colony-forming units (CFU) in relation to a specific unit volume of culture over time is regarded as the most reliable and reproducible method to establish the concentration of viable microorganisms in liquid culture (Davey et al., 2004; Peñuelas-Urquides et al., 2013). This approach is unfortunately laborious, requiring extensive time periods to establish reliable growth metrics, more so in the case of slow-growing organisms such as *M.tb* (Damato et al., 1983; von Groll et al., 2010; Peñuelas-Urquides et al., 2013). The value of such experiments cannot be underrated, as they provide a mechanism with which to understand the impact of genes, growth conditions and energy sources on diversification and growth, enabling a better understanding of tuberculosis biochemistry, infection and eventually treatment (Aldridge et al., 2012).

The ability of organisms to form biofilm structures is a contributing factor to microbial

persistence, allowing pathogenic bacterial populations to survive and flourish, even when exposed to extreme environmental pressures (Costerton et al. 1999). *M.tb* has been reported as being capable of forming drug-resistant bio-films *in vitro* (Ojha et al., 2008; Ackart et al., 2014). This ability is considered to contribute significantly to its pathogenicity, as these biofilms serve to form a protective barrier against antibiotics that are normally active against planktonic bacilli (Esteban and Garcia-Coca, 2018). A hypothetical model for *M.tb* biofilm formation postulates that "dormant" bacilli are arranged around the periphery of lesions close to open airspaces; exposure to oxygen results in reactivation of these bacilli (Orme, 2014). The slower growth rates and lower metabolic activity prevalent in biofilm communities is thought to perpetuate a higher tolerance to therapeutic treatment (Solokhina et al., 2017). Thus, the discovery of drug targets and further development of treatments with potential to attenuate and impede biofilm formation would define a novel mechanism with prospects to considerably reduce anti-TB treatment durations (Ojha et al.,2008; Esteban and García-Coca, 2018).

The complex genetics and biochemistry, coupled with obstacles in infection control has largely driven the success and survival of this complex organism, resulting in a global crisis. A wealth of information has been garnered from epidemiological and basic science studies. The scope of the problem, the control measures currently being implemented and their efficacy (or lack thereof), as well as the development of therapeutic drugs and diagnostic tools to address the disease burden, has been well covered in literature to date.

It is clearly understood that despite good first- and second-line therapeutics being readily available to treat drug-susceptible TB, factors such as latency, an ineffective vaccine, a lack of rapid point of care diagnostic tests, persistence and prolonged chemotherapy regimens exacerbate the situation. This has driven the urgent search for novel drugs with sterilizing ability to target the bacilli and inhibit disease progression, in an effort to shorten the duration of treatment and reduce the risk of relapse (Betts, et al., 2002; Krieger et al., 2012). New drug compounds face the necessity of needing to be equally potent in their ability to sterilize both sensitive and drug resistant strains (Ambady et al., 2012). Unfortunately, in the on-going search for candidate antigens, the

number of potential novel targets to be developed as anti-TB vaccine and therapies remains few and far between (Carabali-Isajar et al., 2018).

In an attempt to bridge this gap and add to the current body of knowledge, this study aimed to establish the effect of inhibiting functionality of the two major adhesin proteins, HBHA and MTP. A novel double gene knockout mutant strain of *M.tb* was generated and confirmed, and used to establish the dual effect of these surface-associated proteins on growth kinetics, viability, biofilm formation, as well as the adhesion and invasion capacity of *M.tb* when infecting pulmonary epithelial cells *in vitro.*

1. Literature review - preface

The review below provides an overview and background to the current infectious disease pandemic caused by *M.tb*. The mechanisms behind infection, the immune responses mounted by the human host as well as current perspectives in therapeutics and diagnostics will be highlighted. The review gives some attention to the primary initiators in infection, the adhesin molecules, and elaborates on the use of molecular techniques in the study of the host-pathogen interactions.

2.1. *Mycobacterium tuberculosis* – the immortal microbe

A disease of antiquity, *M.tb*, the bacillus responsible for TB, emerged as a human pathogen with roots postulated to originate in Africa, from where it was able to colonize the world through the Out-of-Africa migrations of modern *Homo sapiens* (Gagneux, 2012). The tubercle bacillus is phenotypically characterised by its slow growth, conserved genetic homogeneity, complex cell envelope arrangement, intracellular pathogenesis and dormancy traits (Cole et al., 1998). The bacterial population resulting from its widespread global reach is very large. However, the classical members of the *M.tb* complex (MTBC) - *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. canetti* and *M. microti* - continue to have very limited genetic diversity (Frothingham et al., 1994; Richter et al. 2003; Supply et al., 2013). Systematic sequence analysis of multiple loci on independent isolates concluded that the genome of *M.tb* is either relatively evolutionarily young, or is uniquely and unusually inert (Cole et al., 1998).

2.1.1. General morphology

Morphologically, bacilli typically appear under light microscopy as straight, sometimes slightly curved rods, approximately 3-5 µm in length and 0.2-0.6 µm wide (Velayati and Farnia, 2012). With a slow doubling time (18 – 24 h), the individual microbes tend to vary in size and shape (long rods or coccobacilli) based on the general growth conditions and age of the bacterial culture (Flynn et al., 2011; Velayati and Farnia, 2012).

2.1.2. Cell wall structure

The uniquely characteristic thick, lipid-rich cell wall of *M.tb* (composed of long-chain fatty acids, peptidoglycan, proteins and glycolipids) (Fig. 1.) is one of the benchmark features that has contributed to the infectious success of this pathogen (Flynn et al., 2011; Kumar et al., 2013), and is considered to confer an advantage in stressful conditions of osmotic shock or desiccation (Jarlier and Nikaido, 1990). The structurally complex, mycolic acid cell wall positions the cell as practically impermeable to the majority of conventional antimicrobial drugs, limiting the number of treatment agents against TB (Jarlier and Nikaido, 1990; Kumar et al., 2013).

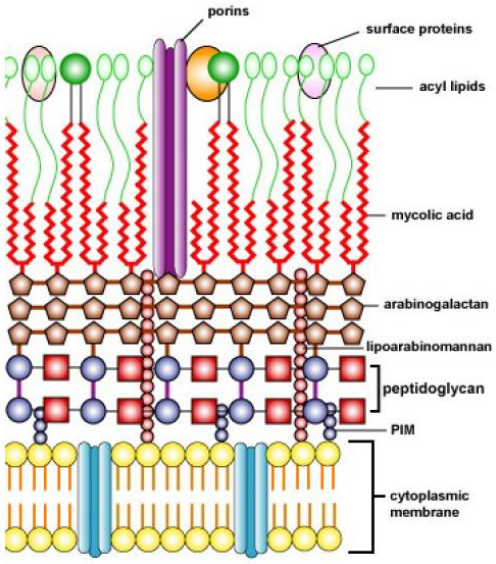

Fig. 1: The *Mycobacterium tuberculosis* cell wall structure: the acid-fast cell wall contains a variety of glycolipids, peptidoglycan and mycolic acids. A layer of polypeptides and mycolic acids (free lipids, glycolipids, and peptidoglycolipids) sits over arabinogalactan/mycolic acid layer. Other glycolipids present include lipoarabinomannan and phosphatidyinositol mannosides (PIM). Porins are also present to enable hydrophilic molecules to move through the outer membrane of the cell wall. Image sourced from: Kaiser, 2018.

2.1.3. Metabolic adaptation

The success of the bacteria can further be attributed to an enhanced metabolic flexibility; the prototrophic tubercle bacillus is able to assemble all its components from basic sources of carbon and nitrogen, while being able to utilize pre-existing synthesized organic compounds as a primary source of energy (Edson, 1951; Ramakrishnan et al., 1972; Niederweis, 2008). This biochemistry is central in enabling the bacilli to effectively adapt to fluctuating environmental changes over the course of infection. Thus, the survival of *M.tb* is not dictated by nutritional quality and physical conditions (as with other infectious bacteria), as these resilient organisms are able to efficiently adapt to stressors such as hypoxia, temperature changes, nutrient deprivation, pH, and a host of other exogenous factors (Velayati and Farnia, 2012).

2.1.4. Evasion strategies

Virulent *M.tb* complex species have established clever strategies to evade or modulate the host immune response to their favour (Forrellad et al., 2013). Most impressive is the ability of the bacterium to circumvent the hostile intracellular environment of the alveolar macrophage upon being engulfed. *M.tb* inhibits phagosome-lysosome fusion to effectively evade the acidic elements inside the phagolysosome, by recruiting host proteins, such as Coronin1, a tryphtophan aspartate containing coat protein (TACO) (Jayachandran et al., 2007). TACO is retained at the phagosomal surface once recruited by *M.tb*., where it activates calcium–calcineurin signalling, further inhibiting lysosome fusion with mycobacterial phagosomes (Fig. 2.) (Jayachandran et al., 2007). Lysosomal hydrolases, reactive nitrogen and reactive oxygen species - which ordinarily destroy intracellular bacteria - are subsequently rendered useless (Forrellad et al., 2013).

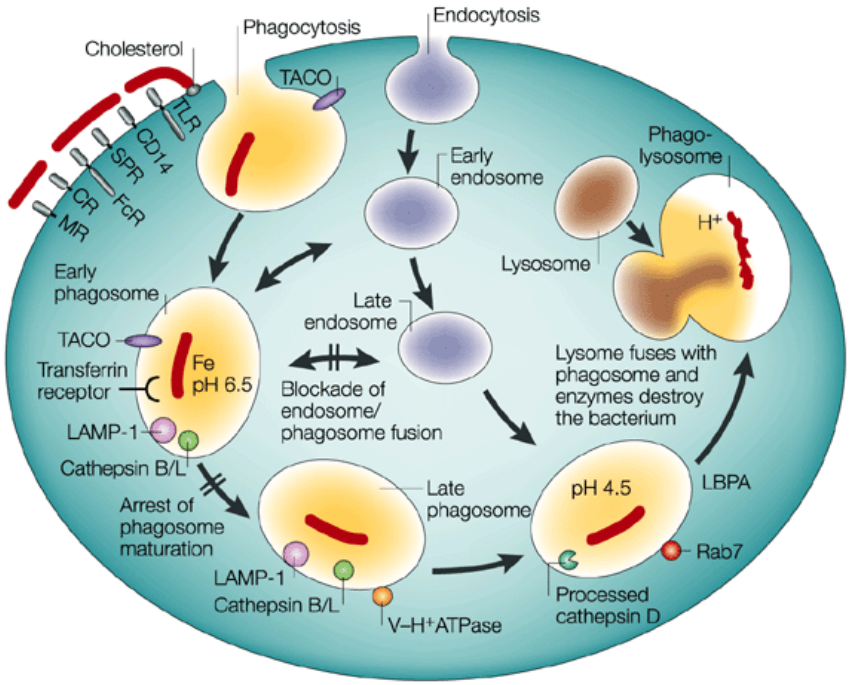

Fig. 2: The influence of *M. tuberculosis* on phagosome and endosome maturation; once engulfed, *M.tb* finds itself in a phagosome, where maturation is arrested at an early stage. The early phagosome-harbouring mycobacteria retains TACO (tryphtophan aspartate containing coat) proteins, which serve to prevent further maturation. *M.tb* inhibits phagosomal acidification with a V–H+ ATPase, preventing fusion with the endosomal pathway. Image sourced from: Kaufmann, 2001.

2.2. The global healthcare crisis: tuberculosis

Previously considered as an inherited condition, TB (the notorious white plague, consumption or phthisis) was defined by Robert Koch in 1882 as one of the first infectious diseases caused by a bacterial pathogen, *Mycobacterium tuberculosis* (Orgeur & Brosch, 2018). TB is considered the ninth leading cause of death worldwide, and is the leading cause of mortality from a single infectious agent, ranking above HIV/AIDS (WHO Global Tuberculosis Report, 2017). During 2017, an estimated 10 million people developed active TB, and 1.6 million succumbed to the disease (WHO Global Tuberculosis Report, 2018). Of those that fell victim to death, 0.3 million were HIV-positive (WHO Global Tuberculosis Report, 2018). The WHO further estimates that 2017 saw over 558 000 new cases reported with resistance to the most effective first-line drug, Rifampicin, of which, 82% were diagnosed as having MDR-TB (WHO Global Tuberculosis Report, 2018). The global incidence in countries with a high prevalence is highlighted in Fig. 3 (WHO Global Tuberculosis Report, 2018), drawing attention to countries such as India, China, Indonesia, the Philippines and South Africa. South Africa ranks seventh amongst the high burden countries for TB ((WHO Global

Tuberculosis Report, 2017). Even though over a century of research has been performed, this non-discriminant agent of infection continues to cause death, despite the ready availability of cheap, efficacious, and curative therapy (Bermudez et al., 2002; Algood et al., 2003; Almedia et al., 2008; Chang et al., 2007).

Fig. 3: WHO presentation of the TB incidence estimate in 2017, for countries with at least 100 000 incident cases. The data places emphasis on the burgeoning epidemic present in Asian countries with a dense population demographic, such as India, China and Indonesia. Image sourced from: WHO Global Tuberculosis Report, 2018.

2.2.1. Drug resistance

The emergence of drug-resistant TB in recent years has contributed to the problem at hand, and is further driven by ineffective or delayed drug-susceptibility testing (Alva et al., 2013). Drug resistance arises when bacteria replicate in the presence of therapeutic drug concentrations that are only partially suppressive, resulting in the formation of mutants able to outgrow wild-type strains (van den Boogaard et al., 2009). Drug resistance to the most efficacious first line TB drugs, isoniazid (INH) and rifampin (RFP), is defined as multi-drug resistant TB (MDR-TB), while the more likely fatal extensively drug-resistant TB (XDR-TB) is classified as MDR-TB with additional resistance to further second line drugs (any fluoroquinolone, and to at least one second-line injectable drug including capreomycin, kanamycin or amikacin) (Shah et al., 2008; Caminero et al., 2010; Alva et al., 2013; Caminero and Scardigli, 2015; Sulis et al., 2016). Fig. 4 implicates 3 major populations (India, China and the Russian Federation) to be the predominant contributors to the current global MDR-TB epidemic, as they

cumulatively account for almost half of the recorded global cases (WHO Global Tuberculosis Report, 2018). Unfortunately, the prevalence of these resistant and extensively drug resistant strains continues to rise (Carabali-Isajar et al., 2018), with 2017 statistics attributing 8.5% of MDR-TB cases as being extensively drug-resistant TB (XDR-TB). The emergence of totally drug resistant TB (TDR-TB) strains, which present with *in-vitro* resistance genotypes/phenotypes to all first and second line drugs (including isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, ethionamide, para-aminosalicylic acid, cycloserine, ofloxacin, amikacin, ciprofloxacin, capreomycin and kanamycin) served as a stark and swift call for countries to mobilize resources to accelerate implementation of proper treatment and monitoring protocols, especially in patients diagnosed as multi drug-resistant (Udwadia, 2012).

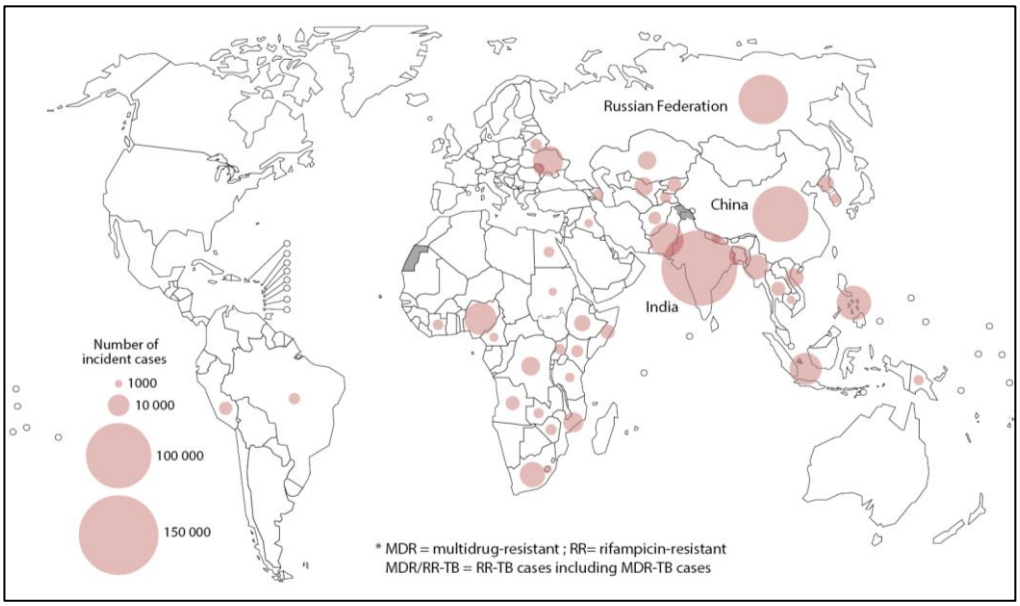

Fig. 4: The estimated incidence of MDR/RR-TB in 2017, for countries with at least 1000 incident cases indicates 3 major populations as the most significant contributors to the rising prevalence of MDR-TB; India, Russia and China. Image sourced from: WHO Global Tuberculosis Report, 2018.

2.2.2. The HIV pandemic

The HIV epidemic has resulted in the prevalence of *M.tb* increasing worldwide, with 2017 statistics indicating a mortality rate of almost 300 000 people dying of HIV-associated TB (WHO Global Tuberculosis Report, 2018). Alongside these fatalities, a further 900 000 new TB cases were recorded amongst the global HIV-positive population, of which 72% were reported out of the African continent (WHO Global Tuberculosis Report, 2018) (Fig. 5). South Africa's current global position is largely

driven by the ever-increasing rates of HIV co-infection coupled with a high incidence of multi- and extensively drug resistant strain outbreaks (Calligaro and Dheda, 2013; O'Donnell et al., 2013; Calligaro, et al., 2014). In 2015, the average TB/HIV co-infection rate across South Africa was 56.7%, with patients presenting with the highest co-infection rates in Gauteng (68.4%), Mpumalanga (68.1%) and KwaZulu-Natal (63.6%) (Van Leeuw and Loveday, 2015). Lower rates of TB/HIV co-infection were detected in The Eastern Cape (45.2%), Northern Cape (41.1%), Western Cape (38.5%). Durban, KwaZulu-Natal is regarded as the epicentre of the country's TB/HIV co-infection and the epidemic is staggeringly widespread across the province, into the most far-reaching parts, with TB/HIV co-infection rates touted as being the highest in the country in the notorious uThukela district (71.2%) (Van Leeuw and Loveday, 2015).

Epidemiologic data indicate HIV-infected individuals are much more susceptible to the disease than their healthy counterparts (Goletti et al., 1996). The effect of co-infection on mortality on South Africans in the province is highlighted by a hospital post mortem-study of young inpatients (18-45 years) that found 94% of patients to be HIV+ and 69% of them to have active TB or be on TB treatment at the time of death (Cohen et al., 2010). This synergistic relationship combined with outside factors such as poverty, under-nutrition, diabetes, drug use and smoking can be held responsible for the overwhelming resurgence of TB as a serious global public health epidemic, one that is further exacerbated by a significant increase in the prevalence of multi-drug resistant strains (Ambady et al., 2012; WHO Tuberculosis Report, 2017). The significant effects of TB/HIV co-infection can be seen in the impact being made medically, socially and economically (Skvortsov and Azhikina, 2012).

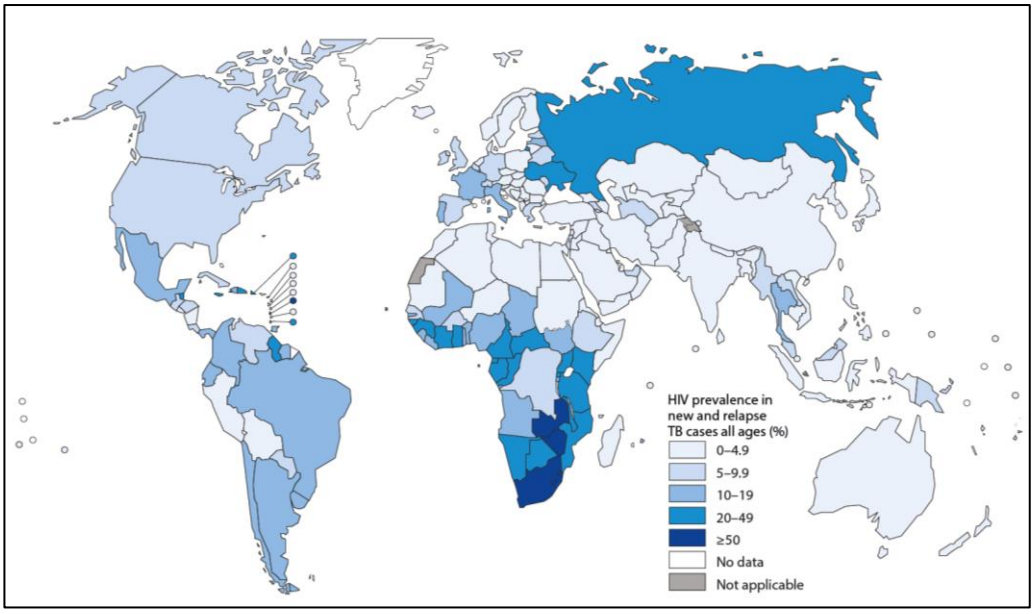

Fig. 5: Estimates of HIV prevalence in new and relapse TB cases – within 2017, 900 000 new cases of TB were reported within the global HIV-positive population, with 72% of these cases reported out of Africa. Image sourced from: WHO Global Tuberculosis Report, 2018.

2.3. Engaging the host: mechanisms of infection and the immune response

2.3.1. Mechanism of infection

M.tb host infection occurs by inhalation of aerosolized droplets of proteinaceous pulmonary secretions carrying infectious, live bacteria (Bermudez & Goodman, 1996). Inhalation of a very low dose of bacilli (usually 1–5 tubercle bacilli) is thought to be sufficient for infection to occur (Balasubramanian et al., 1994). Once the bacteria become seeded within the respiratory tree, the droplet nuclei (ranging between 5 and 10 µm) localize within the alveoli (Volk et al., 1986).

Bacilli interact with both the epithelial cells lining the alveoli and the target host cells (alveolar macrophages) (Bermudez & Goodman, 1996). Emerging evidence indicates that the alveolar epithelium, and the abundant pulmonary epithelial cells (considered to be the initial barrier faced by *M.tb* upon its entry into the human host) serve as a potential reservoir for the organism and play a key role during progression to active disease (Pessolani et al., 2003; Scordo et al, 2017). Macrophages and dendritic cells become direct targets for infection and activation (Kapoor et al., 2013). The well-documented *M.tb* infection model designates the alveolar macrophage as the primary host cell, where bacilli survive by disrupting the phagocytic cells' bactericidal mechanisms and replicating in an intracellular phagosomal compartment (Bermudez &

Goodman, 1996; Harriff et al., 2014). This process is depicted in Fig. 6a, and is followed by the host immunity attempting to contain the infection by means of granuloma formation, as illustrated by Nunes-Alves et al. (2014) (Fig. 6b).

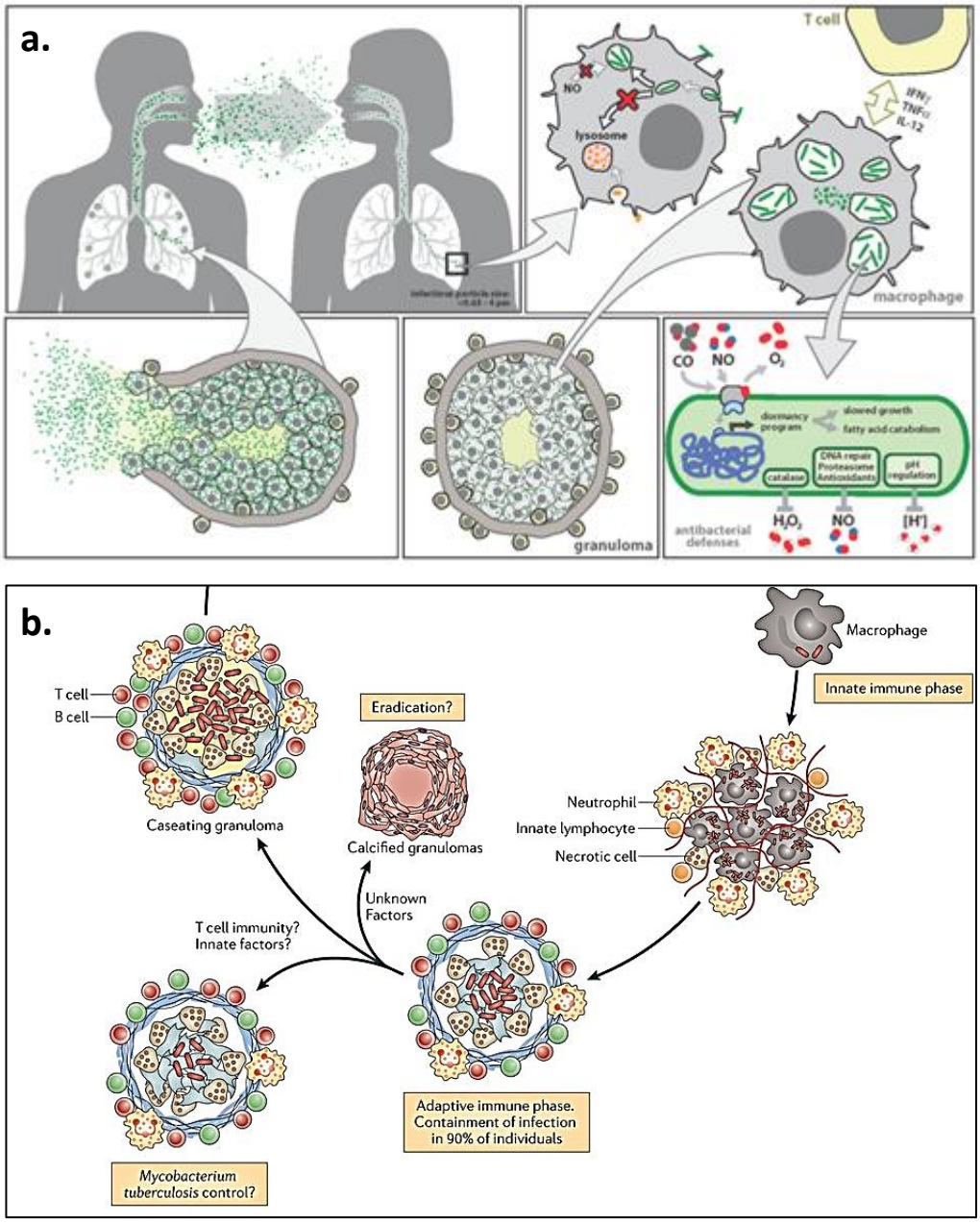

Fig.6: a. Model of TB infection indicating aerosolization of bacilli and infection of macrophages, eventually resulting in granuloma formation. Image sourced from: Shiloh, 2018. b. TB granuloma formation to control and contain infection – granulomas may calcify to eradicate infection or caseate to burst and release dormant bacilli resulting in reactivation. Image sourced from: Nunes-Alves et al., 2014.

1.3.2. Role of macrophages

Infection of the alveolar macrophage is considered the formative step resulting in bacterial dissemination, granuloma formation and initiation of TH1-type immunity (Flynn et al., 2011; Silva et al., 2012). Activated dendritic cells initiate an immune cascade, by presenting the processed antigens to CD4 T-cells (Dreher and Nicod, 2002). Activated lymphocytes and infected macrophages respond rapidly to inflammatory chemokine and cytokine signals, migrating to the site of infection to form organized granulomas in which *M.tb* remains contained, entering into a drug-resistant state of dormancy (Kapoor et al., 2013). The infection is normally contained in the lung by formation of granulomas, where activated macrophages and other immune cells aggregate at the site of infection to restrict mycobacterial dissemination from the lung and limit tissue damage (Saunders et al., 1999; Smith, 2003). In the granuloma structure, some bacteria remain essentially dormant and contained for decades without any presentation of active clinical disease (latent TB) (Silva et al., 2012; Kapoor et al, 2013). However, in the event that the immune system becomes compromised, the caseous granuloma can become unstable and rupture, releasing contained dormant bacteria able to reactivate, replicate and spread into the lung and other tissues to cause active TB (Smith, 2003; Kim et al., 2010; Forrellad et al., 2013). The role of alveolar macrophages in TB pathogenesis has thus, been extensively studied and is well documented (Fenton et al., 1996; Keane et al., 1997; Keane et al., 2000; Bell and Noursadeghi, 2018; Esmail et al., 2018).

2.3.3. The role of epithelial cells

In contrast to macrophages, the interaction between *M.tb* and the lung epithelial cells has not been studied as extensively, even-though alveolar Type II pneumocytes and airway epithelial cells (AEC) initiate early defense mechanisms upon exposure to *M.tb* (Hariff et al., 2014). Although the primary mechanism of TB infection is via macrophages, within alveoli epithelial cells are present in far larger numbers than macrophages (Crandall and Kim, 1991). Additionally, these cells are able to mount a defense against infection via production of molecules such as cytokines, chemokines and antimicrobial B-defensins, surfactants, NO and other molecules that are able to enhance the anti-microbial function of infected macrophages, or kill *M.tb* directly (Roy et al., 2004; Sharma et al., 2007; Lee at al., 2009). Given the large number of epithelial cells (28000 type I and 1400 type II) relative to the limited number of alveolar

macrophages (between 50 and 100) present within the alveolar space, invading bacilli have a greater chance of survival within epithelial cells than in the harsh environment presented by the presence of unarrested phagosomes within the macrophage cytoplasm (Bermudez & Goodman, 1996; Ashiru et al., 2010).

Mycobacteria are able to effectively invade and replicate both in macrophages and in epithelial cells located in the alveolar spaces of the lung (Li et al., 2012). Lung epithelial cells present a potentially ideal environment for infecting bacilli to replicate away from the bactericidal mechanisms mounted by macrophages in relative safety (Castro-Garza et al., 2002). *M.tb* is versatile in that it is capable of adhering to, invading and replicating within these pneumocytes *ex vivo* (Bermudez and Goodman, 1996; Mehta et al., 1996). Additionally, epithelial cells present a niche within which the bacilli are provided direct access to the host lymphatic and vascular systems (effected by a disruption and destruction of the alveolar vascular endothelium), without the need for carrier macrophages (Castro-Garza et al., 2002; McDonough and Kress, 1995; Dobos *et al.*, 2000).

2.3.4. Host immune responses

Healthy individuals mount a complex and multi-factorial immune response to *M.tb* infection. Studies of the immune responses in infected lungs are of key interest due to the localized interactions within the organ which serve as primary infection control measures (Algood et al., 2003). The initial *M.tb*-epithelial cell interaction may potentially facilitate the recruitment of macrophages to the site of infection by secretion of chemokines (Alteri, 2005). Infection of host macrophage cells by *M.tb* bacilli leads to the initiation of a cascade of defined inflammatory events, primarily regulated by the production the inflammatory molecules IFN-γ and IL-2 by activated T-cells (Cooper, 2009; Sasindran and Torrelles, 2011). This encourages the release of inflammatory cytokines, such as tumor necrosis factor-α(TNF), interleukin-(IL)-12, IL-6, IL-10 and IL-23 along with a variety of chemokines including (C-C) motif ligand 2 (CCL2), CCL4, CCL5, and (C-X-C) motif ligand 8 (CXCL8) from infected alveolar and tissue macrophages (Algood et al., 2003; Sasindran and Torrelles, 2011).

The factors that enable *M.tb* bacilli to persist in the presence of a strong immune response and that allow most people to control the initial infection remain poorly

understood. Rough estimates predict that only 5–10% of persons infected with *M.tb* actually present with active disease, whilst the vast majority of those infected contain, but do not eliminate and clear the initial infection (Algood et al., 2003). These latently infected individuals do not present with clinical symptoms of disease and are not contagious. However, infection may reactivate to active TB if their immunity becomes severely compromised, as in the case of HIV infection.

2.4. Vaccines, diagnostics and therapeutics - current perspectives

2.4.1. Vaccines

The vaccine Bacille Calmette–Guérin (BCG) came into being almost 100 years ago, after Albert Calmette (1863–1933) and Camille Guérin (1872–1961) passaged *Mycobacterium bovis* over 230 times every 14 days, on potato slices soaked in ox gall (Kaufmann et al., 2017). This painstaking task, which began in 1906, was an attempt to generate an attenuated vaccine against *M.tb*. Even though they had preliminary evidence for the safety and the protective capacity of their vaccine candidate in 1913, they continued on until the 230th passage before using animal models to verify its efficacy and safety (Kaufmann et al., 2017). To date, BCG continues to be the only licensed vaccine against TB. The vaccine is able to confer protection against extra-pulmonary infection in infants (including meningeal infection), but is ineffective in preventing pulmonary TB (Cernuschi et al., 2018). Additionally, it must be stressed that the efficacy of the BCG vaccine remains complicated by the fact that BCG is not derived from a single, pharmacologically well-defined vaccine but is comprised of a pool of different BCG daughter strains (Oettinger et al., 1999). These strains have acquired phenotypic and genotypic variations as a result of ongoing sub-culturing across different laboratories (Bottai and Brosch, 2016). In a study performed by Zhang et al. (2016), 13 different BCG strains were compared in phenotypic and genotypic analysis - distinct levels of virulence, potentially linked to strain-specific duplications and deletions within genomic regions, were recorded across the various strains.

The absence of a comprehensive and effective vaccine against pulmonary TB continues to be a major roadblock in treatment of pulmonary TB (Russell et al., 2010). This remains largely due to the duration it takes to move candidates through each stage of the development pipeline before a vaccine may become available and approved for

human use. As highlighted in the Global Report on Tuberculosis Vaccines (2018), within the current TB clinical and pre-clinical pipeline, fourteen TB vaccine candidates are currently in clinical trials (Fig.7). Of these candidates, only one vaccine, the Chinese candidate, Vaccae™, has entered Phase 3 testing. Late Phase 2 testing is underway on three vaccines, while ten are in earlier stages of assessment. An additional seven potential vaccines are in pre-clinical trials, and twenty novel vaccine strategies in discovery and development.

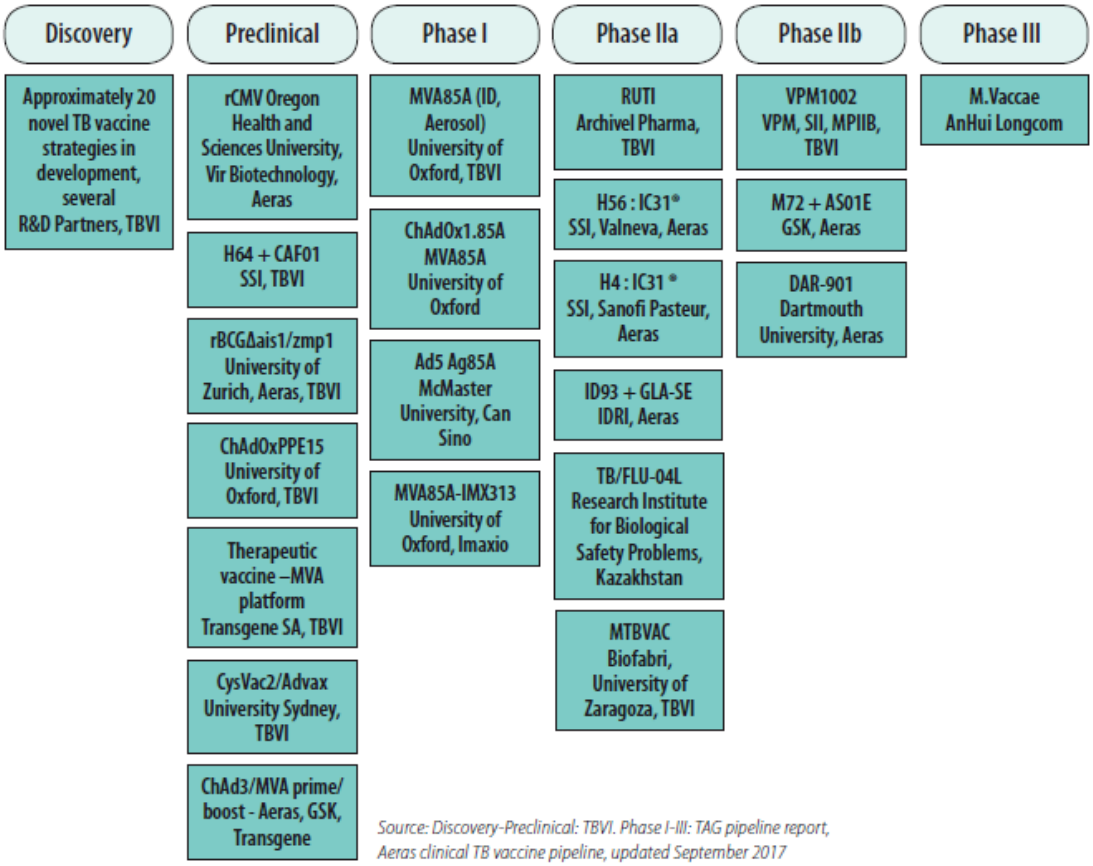

Fig. 7: The current clinical and pre-clinical TB vaccine pipeline compiled using data provided by TBVI, Aeras and TAG. Image sourced from: Global Report on Tuberculosis Vaccines 2018 Executive Summary.

2.4.2. TB diagnostics

Sputum smear microscopy is still heavily relied upon by many high-burden countries, although diagnostic technology has advanced considerably, with only 57% of global TB cases of bacteriologically confirmed, and even less undergoing drug susceptibility testing (Cox & Nicol, 2018). Although more sensitive and specific diagnostics are available, such as Xpert MTB/RIF automated rapid molecular assay (Cepheid, USA),

the high cost factor in comparison to other commonly implemented laboratory methods has restricted implementation to high burden regions (Kelamane et al., 2015; Cox & Nicol, 2018**).** The abilities to detect TB early and provide effective and continuing care in such populations are essential to curtail further transmission (Dara & Zachariah, 2018).

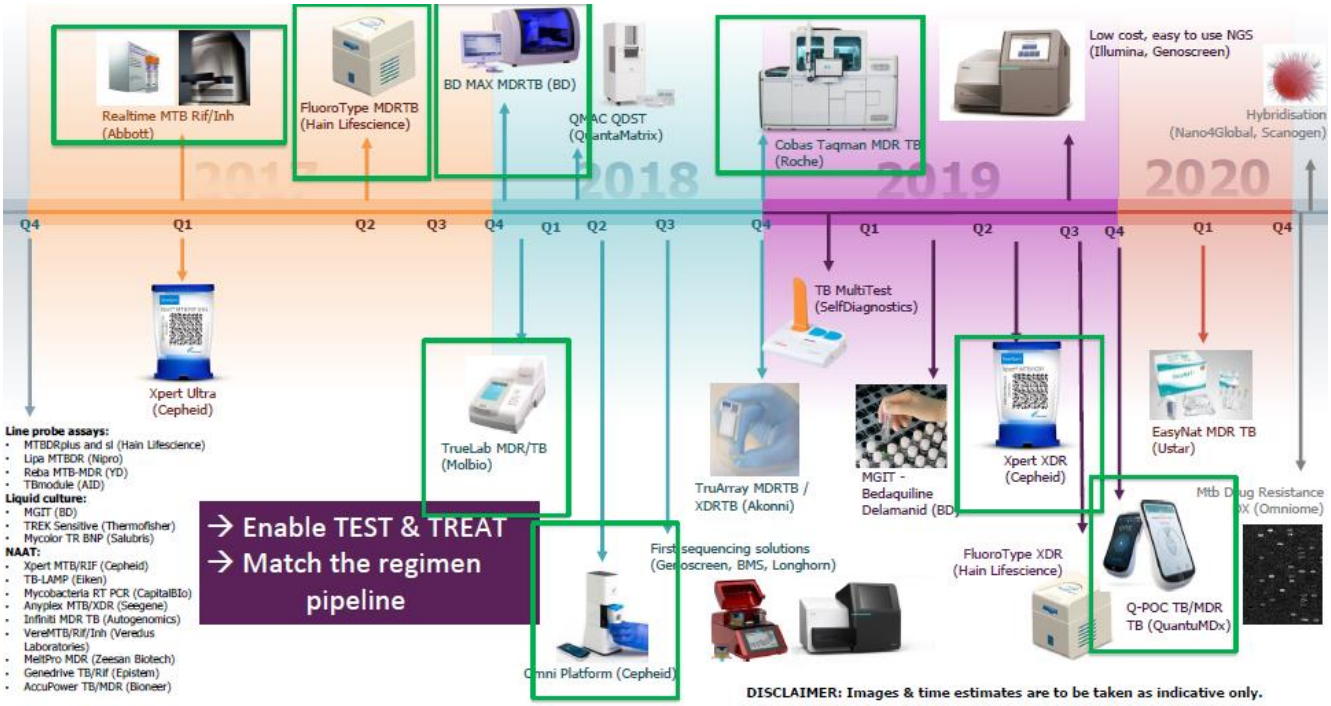

Fig. 8: TB diagnostics pipeline showing recent and future POC devices. Image sourced from: Denkinger, 2017.

Currently, a range of phenotypic, serological and genotypic methods are applied in the diagnosis of *M.tb*. These methods include the abovementioned GeneXpert MTB/RIF automated rapid molecular assay, the Determine TB-LAM assay, MTBDRplus and MTBDRsl line probe assays (Hain Life Science), the Qiagen QuantiFERON®-TB Gold test interferon gamma test, other nucleic acid amplification tests (NAATs), traditional liquid/plate culture and drug susceptibility testing methods (Fig. 8). These all have their own pitfalls and advantages and thus, largely fall short in delivering a tool that meets the criteria set by the WHO in defining a comprehensive point of care diagnostic, outlined in Table 1. (World Health Organization, 2014, García-Basteiro et al., 2018).

Table 1: High priority target product profiles in development of an ideal TB point of care (POC) diagnostic tool, as defined by the WHO (2014) – adapted from García-Basteiro et al., 2018

✓ Able to detect both pulmonary and extrapulmonary tuberculosis (TB)
✓ Can be effectively used in TB presumptive cases with children, adults, HIV-positive or HIV-negative
✓ A range of easily accessible body samples can be applied in testing
✓ Can be easily used by health care workers with minimal training
✓ Peripheral or rural community health facilities can apply the method successfully
✓ The method can be performed throughout a broad range of temperature and humidity
✓ Results are available in less than 20 minutes
✓ Little to no maintenance required
✓ Affordable – cost equivalent or less than 4 US $ per test
✓ High sensitivity - similar to that of GeneXpert MTB/RIF for both pulmonary and extrapulmonary TB (if intended for use as a triage test, outcomes must be 95% comparable to culture results)
✓ High specificity - similar to that of GeneXpert MTB/RIF for both pulmonary and extrapulmonary TB (if intended for use as a triage test, outcomes must be 80% comparable to culture results)

As much as the most sensitive method of detecting *M.tb* remains bacterial culture (Dinnes et al., 2007, World Health Organization, 2011; Ryu, 2015), the method is cumbersome and drawn out, as bacilli take between 2-6 weeks to grow on solid culture media for interpretation. This method often requires the use of antibiotic-containing media, and is prone to risk of contamination due to the prolonged incubations (Nema, 2012). Even so, the BACTEC microbial growth indicator tube (MGIT) liquid culture system remains the fastest form of culture system, producing results within 14-17 days (Dinnes et al., 2007; Gomathi and Kumar, 2014) with a limit of detection ~10–100 cfu/ml versus that of molecular methods, which must detect ≤10 genomic copies of *Mycobacterium tuberculosis* (MTB) DNA (Reed et al., 2017).

The Determine TB-LAM assay (which diagnoses TB by detecting the presence of the *M.tb* antigen lipoarabinomannan (LAM) in urine) has a lower sensitivity when compared to the Cepheid GeneXpert molecular assay, even-though it is an attractive point-of-care option due to its rapid turnaround time to result, ease of use and low cost (Lawn et al., 2013). The QuantiFERON®-TB Gold test test, which quantifies interferon gamma production in response to both the Culture Filtrate Protein (CFP)-10 and the six kilodalton Early Secreted Antigenic Target (ESAT-6), although affordable, quick and easy to use, has the downfall of showing cross-reactivity to other *Mycobacterial* species (including *M. marinum, M. sulzgai* and *M. kansassi*) (Helmy et al., 2012). Within the domain of NAATs, the Hain Genotype® MTBDR*plus* strip test facilitates the detection

of *rpoB*, *inhA* and *katG* mutations, detecting *M.tb* infection amongst the presence of other infecting agents, such as HIV, while defining infection with drug resistant strains (Hillemann et al., 2007; Barnard et al., 2008). Additionally, the Hain Genotype® MTBDR*sl* is a rapid DNA-based test that enables detection of specific mutations linked to resistance to fluoroquinolones and second-line injectable drugs (SLIDs) (Theron et al., 2016).

Even though research and development in the field of point of care tools and novel targets for diagnosis have taken off in recent years, most of the currently available TB point-of-care diagnostic tools lack one or more of the desirable WHO Target Product Profiles criteria (as per Table 1) to allow implementation in a rolled-out strategy (García-Basteiro et al., 2018). Innovative technologies able to provide effective early detection at an affordable price for low-income and rural settings remain few and far between. However, the commercially-available POC tests that are available, offer value in trying to gauge a better understanding of infection and control, thereby paving the way for development of a more applicable and effective tool for implementation within high-burden settings (García-Basteiro et al., 2018).

2.4.3. Anti-TB therapeutics

As a standard first line regimen, four drugs are administered at therapeutic doses (including isoniazid, rifampicin, ethambutol and pyrazinamide) under 'directly observed treatment short-course (DOTS), a process that is implemented to ensure treatment compliance by monitoring administration of anti-tuberculous therapy under regular supervision (Pollett et al., 2016). MDR-TB treatment necessitates the use of additional second-line drugs, that are more expensive, less effective and more toxic than first-line regimens (Center for Disease Control and Prevention, 2006).

Drug treatment of MDR-TB as stipulated by the WHO guidelines, recommend the combined use of at least four different drugs. Typical regimens include pyrazinamide with numerous second-line drugs (Falzon et al., 2011; Park et al, 2015). The remaining three drugs must comprise: ethionamide, a later-generation fluoroquinolone or prothionamide; an injectable parenteral agent (kanamycin, amikacin, or capreomycin), and one of either cycloserine or p-aminosalicylic acid (PAS) (Falzon et al., 2011). On average, only 54% of patients presenting with MDR-TB are treated successfully, with

diagnosis often being delayed or undetected (Dara & Zachariah, 2018). Extensively drug-resistant TB (XDR-TB) has far worse treatment outcomes (40% on average, but as low as 19% when the disease is perpetuated by strains with resistance phenotypes indicative of TDR-TB) (Sulis et al., 2016). The lengthy treatment regimens (18–24 months) and higher number of drugs used for treatment in comparison to drug susceptible TB treatment (often causing severe and adverse side-effects) are considered to be key factors leading to treatment failure (Dara & Zachariah, 2018, Yang et al., 2017).

One of the most common reasons behind the lack of success in the initial anti-TB regimen in high-burden, resource poor settings, has been the inability to deliver a complete treatment to a patient. The shortage of drugs to treat the more commonly prevelant MDR- and XDR-TB strains in these zones further exacerbates the issue (Mwinga, 2001). Following from a 50-year drought without a new TB drug being released, 2012 saw the approval of two new medicines, delamanid and bedaquiline (by the US Food and Drug Administration (FDA) and by the European Medicines Agency, respectively) (Zumla et al., 2014; Gandhi et al., 2018; Huerga et al., 2018). This followed from the successful treatment of MDR-TB patients in clinical trials using TMC207 (bedaquiline) and OPC-67683 (delamanid) (Diacon et al., 2009; Gler et al., 2012; Gandhi et al., 2018). South Africa has recently become the first country in the world to provide bedaquiline as part of the country's national care regimen for people living with multidrug-resistant tuberculosis (MDR-TB), aiming to reduce longer treatment programs that typically make use of injectable drugs. Similarily, treatment plans for XDR-TB are currently being addressed in a clinical trial evaluating a novel regimen of linezolid, bedaquiline and pretomanid (an experimental drug from the same class as delamanid) (Gandhi et al., 2018). The preliminary findings as reported by The Global Alliance for TB Drug Development highlight a treatment regimen of only 6–9 months, a surprising contrast to the recommended 24-month course. The WHO has encouraged the use of both these new drugs by creating guidelines in support of the 9-month shortened MDR-TB regimen (Falzon et al., 2017).

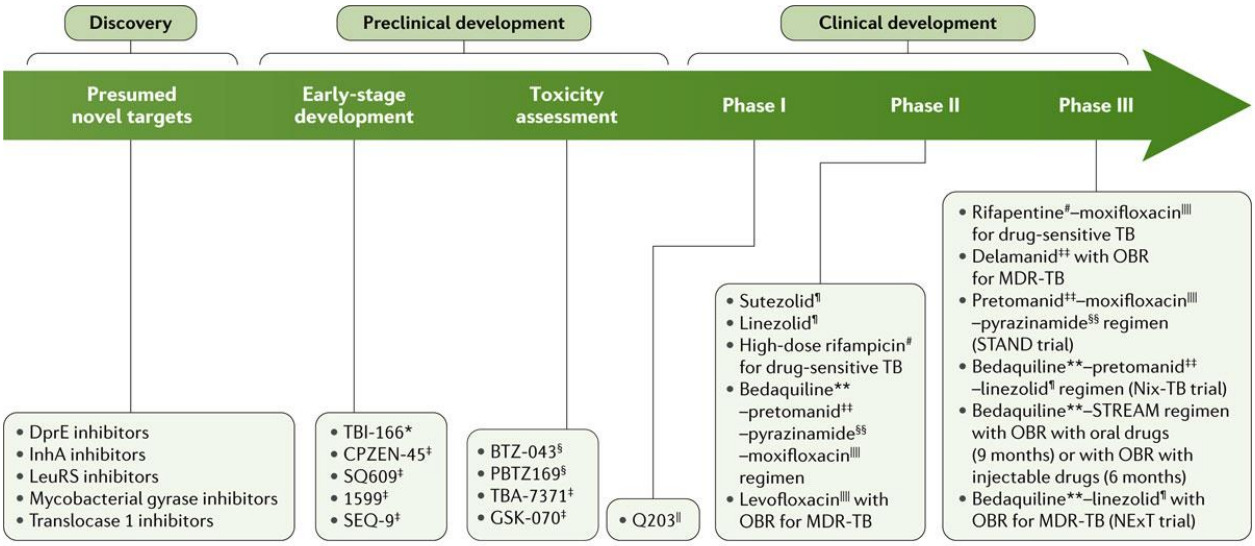

Fig. 9: The current TB drug pipeline, highlighting drug leads, those in early development and candidates currently in Phase1,2 and 3 trials. Image sourced from: Working Group for New TB Drugs, adapted by Pai, et al. (2016).

The New Drugs Working Group of the Stop TB Partnership compiles a pipeline based on independent feedback from drug development entities, giving focus to drugs in discovery, pre-clinical development and clinical development (Fig. 9). Within the clinical development phase, a combination of ten novel and recycled compounds are under evaluation in phase II trials or as a component of anti-tuberculosis (TB) drug regimens (Working Group for New TB Drugs, 2018). The phase II and phase III trials generally aim to combine these compounds in regimens that are significantly shorter and easier to implement, targeting therapy at individuals with first-line drug resistant TB (Pai, et al., 2016).

TB treatment regimes, if successfully implemented according to recommendations, should reduce transmission and thus the number of individuals presenting with active TB (Cox & Nicol, 2018). However, the slow decline in disease burden along with an inadequate response to the surge of drug-resistant TB suggests a profound failure of public health globally, implying that TB programs, while reducing deaths, are insufficient to overcome other drivers, such as poverty, famine, political instability, migration, the global HIV pandemic and drug/alcohol abuse (Cox & Nicol, 2018; Millet et al, 2013, Naidoo, et al., 2007).

2.5. Adhesins: potential drug and vaccine targets

To survive and persist within the host, *M.tb* synthesises and recruits a diverse range of biomolecules (Lamichhane et al., 2011; von Both et al., 2018). These biomolecules are often obtained from the host extracellular/ cellular environment but may also be synthesized internally (e.g. ESAT6 and CFP10, which actively enable the bacilli to evade the phagolysosome) (Lamichhane et al., 2011; von Both et al., 2018). Considering the small genome sizes of bacteria in general, the ability of proteins to carry out multiple functions allows pathogenic organisms to effectively maximize their limited genetic information (Dumke et al., 2011). Identifying and characterising these persistence-facilitating molecules and establishing how they engage with human host components is an attrative prospect, due largely to the impact made by this assiduous pathogen on global health (Glickman & Jacobs, 2001).

To develop effective therapeutic measures targeting the initial interaction between host cells and the pathogen, the appropriate proteins involved need to be identified, characterized, classified and their roles established. As with many pathogenic micro-organisms, adhesion of the infecting agent to the host surface is thought to be a crucial prerequisite in the pathogenic process of TB infection, allowing for host colonization to be established (Barbosa et al., 2006; Squeglia et al., 2017). Mycobacteria display multiple surface proteins with primary and secondary adhesin function that interact with receptors on host cells (Menozzi et al., 2006). Many reviews have recently discussed these protein molecules in great detail, highlighting the alternative primary roles that some have, while discussing the mechanisms of adhesion to the bacterial surface and how they interact with host cells (Kumar et al., 2013; Govender et al. 2014; Ramsugit and Pillay, 2016). This information positions adhesin proteins as valuable pharmaco-therapeutic targets, as most adhesins play important roles in the life-cycle of mycobacteria and are core virulence factors (Squeglia et al., 2017).

2.5.1. Heparin binding hemagglutinin adhesin

Previously, the only adhesin/invasin described definitively for *M.tb* was the heparin-binding haemagglutinin adhesin (HBHA). This is a 28-kDa surface protein virulence factor that facilitates the dissemination of *M.tb* from the site of primary infection by facilitating engagement with epithelial cells (Menozzi et al., 2006; Esposito et al.,

2011). Pethe et al. (2001) employed an HBHA-deficient strain to show a significant reduction in the adhesion to and invasion of A549 pneumocytes, when compared to the wild-type. This study resulted in HBHA being regarded as the principal *M.tb* adhesin prevalent in its interaction with epithelial cells (Pethe et al., 2001). *hbhA* mutant strains show a reduced ability to bind epithelial cells and disseminate from the lungs to other tissues (Menozzi et al., 2006; Esposito et al., 2011). The two central steps in TB pathogenesis, namely bacterial aggregation and cell adhesion, are facilitated by HBHA (Esposito et al., 2012), with its effect on aggregation in *M.tb* visible in studies by Menozzi et al. (1996) (Fig. 10). This adhesin has also been shown to provide strong protection against *M.tb* in *in vivo* mouse models and is touted as a vaccine candidate against TB with great potential (Hougardy et al., 2007). As a diagnostic antigen, HBHA is also a marker to distinguish latent from active TB in humans as the presence of HBHA induces higher levels of IFN-γ in latent infection (Hougardy et al., 2007).

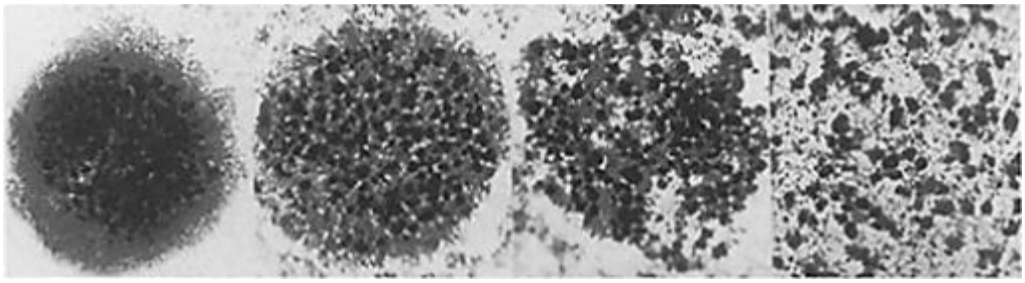

Fig. 10: HBHA-mediated aggregation of *M.tb*, effectively induced by incubating 108 bacteria/ml with final concentrations of HBHA proteins purified from H37Ra. Images (left to right) indicate incubations with 5, 1.25, 0.15 and 0 µg protein/ml (final concentration). Image sourced from Menozzi et al. (1996).

2.5.2. *M.tb* curli pili

Pili (threads) are one of the most commonly found forms of bacterial adhesins, and interact with glycoprotein and glycolipid receptors localized on host cells (Esko & Sharon, 2009). These hydrophobic adhesion molecules are used by bacterial pathogens to initiate infection of host cells (Finlay & Falkow, 1997), with the adhesin tip aiding in their structural, virulence-associated functions (Telford et al., 2006). *M.tb tuberculosis* pili proteins (MTP) are produced during pathogenic adherence to epithelial cells (Fig. 11) and play a key role in stimulating the host humoral immune response (Alteri et al., 2007).

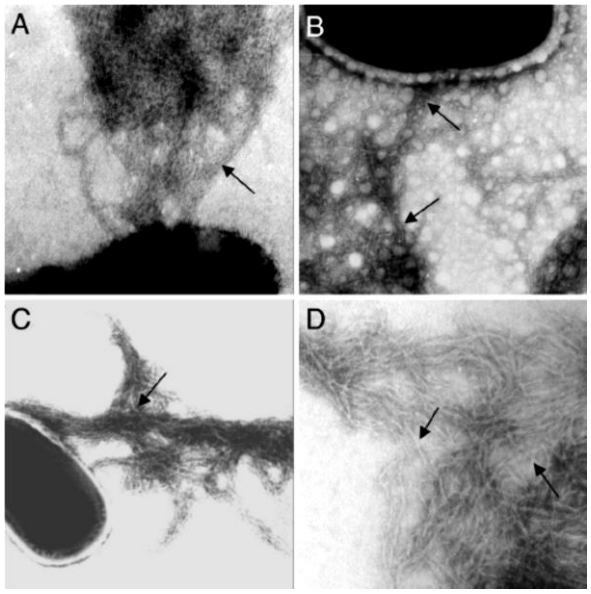

Fig. 11: Transmission electron micrographs captured by Alteri et al. (2007) demonstrate clear production of pili by *M.tb* strains - A. H37Rv, B. avirulent H37Ra, C. clinical isolate CDC155, D. purified MTP. MTP pili fibres are indicated with black arrows. (Mag: 45,000x). Images sourced from Alteri et al. (2007).

Their role as a key adherence factor was cemented by studies that highlighted their involvement in cellular aggregation resulting in biofilm formation (Ramsugit et al., 2013), as well as in the adhesion to and invasion of THP-1 macrophages (Ramsugit & Pillay, 2015) and A549 pulmonary epithelial cells (Ramsugit et al., 2016). The *mtp* gene sequence was also confirmed to be unique to *M.tb* complex strains and highly conserved amongst clinical isolates, establishing it as a novel and fitting biomarker for development as a TB diagnostic test (Naidoo et al., 2014). Naidoo et al. (2018) further highlighted the diagnostic suitability of MTP in designing a POC TB test to screen large patient pools; screening of TB positive and TB/HIV co-infected serum/plasma samples using a synthetic MTP peptide confirmed MTP expression during both TB infection, and TB/HIV co-infection.

Several studies have confirmed the presence of more than one single major adhesin in *M.tb*, which may account for the successful pathogenicity of this organism (Kumar et al., 2013; Esparza et al., 2015; Ramsugit et al., 2016). More recently, Govender et al. (2014) reviewed this class of proteins, highlighting the moonlighting capabilities of key proteins in the biochemical and glycolytic pathways to present themselves for secondary use as adhesin molecules.

2.6. Bridging the gap: Applying molecular techniques in studying host-pathogen interactions

An understanding of the genetic basis of TB drug resistance, host immune evasion and bacterial phenotype is key in developing new diagnostic, treatment and preventive strategies (Grandjean et al., 2017). A critical challenge in drug development is the identification of targets central to the survival of *M.tb* that are either completely absent or significantly divergent, in the host organism (Bellinzoni & Riccardi, 2003). Rapid technological advancements in the molecular tools available for genetic and proteomic studies in recent years has allowed for a greater intensity of research to be undertaken. To date, the currently available anti-tubercular drugs that effectively challenge infections target only a limited number of essential functions in the cell (Sassetti et al., 2003).

Application of molecular tools such as quantitative real-time polymerase chain reaction (qRT-PCR), microarray and whole genome/transcriptome sequencing, in conjunction with *in vitro* growth conditions mimicking the fluctuating environment within macrophages or granulomas (i.e. oxygen deprivation, nutrient starvation and iron limitation) allows for novel genes to be identified for potential application in vaccine or drug development (Zvi et al., 2008).

2.6.1. Transcriptional techniques

The development of techniques such as qRT-PCR, microarray, hybridization and RNA sequencing have allowed for the transcriptional responses of both host and pathogen to be explored during the infection process. Transcriptional techniques such as these, allow the interactions between host and pathogen to be followed and characterized throughout the process of infection, identifying and categorizing pathogenic mechanisms used by *M.tb* to modulate and navigate the immune response (Waddell & Butcher, 2007). The transition of bacteria through variable stages of active multiplication, dormancy and resuscitation can be better understood by means of transcriptomic studies carried out directly on granulomas in lungs, within phagocytized bacteria located in activated macrophages or in *in vivo* animal infection models (Zvi et al., 2008). The understanding of mycobacterial interactions with the host and the subsequent bacilli response has been enhanced by RNASeq expression studies carried

out in conjunction with human patient samples, *in vitro* tissue culture models, models of *M.tb* infection using murine or human derived macrophages and animal models (e.g. murine model of disease) (Waddell & Butcher, 2007).

Expression analyses have the power to elucidate how infecting bacilli respond to the host environment, giving a holistic understanding of the metabolic profile of infecting bacilli, within the different niches that mycobacteria occupy and how they can adapt (Waddell & Butcher, 2007). *In vitro* gene expression studies carried out under conditions which mimic dormancy and/or reactivation provide a vast reservoir of information, and could potentially guide the development of new treatment regimens focussed on targeting dormant cells to reduce later relapse. (Caño-Muñiz, et al., 2018; Zvi et al., 2008). Development of post-exposure vaccines primarily assesses gene products identified as participating in adaptation of *M.tb* to the intracellular habitat as the bacilli transition from replication to dormancy, or resuscitate from dormancy (Zvi et al., 2008).

2.6.2. Genome sequencing

The practice of genome sequencing of mycobacterial strains combined with data from whole-genome DNA microarray and proteomics have been a focus in previous years (Coppola and Ottenhoff, 2018; Walzl et al., 2018). The whole genome sequencing of the H37Rv strain of *M.tb* provided a wealth of data regarding the proteome of this infectious organism, and provided new insights into core biochemical and physiological processes facilitating the survival of the bacilli (Cole et al., 1998; Tekaia et al., 1999b). The availability of the whole genome sequence of *M.tb* has placed TB drug research in a favourably advantageous position by providing genetic and bioinformatic tools that may be used to further study the potential of prospective drug targets for success (Bellinzoni & Riccardi, 2003). This milestone in TB research has paved the way for the use of advanced bioinformatics procedures, both in genomics and transcriptomics research, and has allowed for structural and functional information of critically important survival components whose biological roles were unknown or obscured to come to light (Tekaia et al., 1999b). The genome analysis of *M.tb* defined the major importance of lipid metabolism to the survival of the organism, and exposed the existence of two novel protein families, PE and PPE, with unusual amino acid

sequences (Tekaia et al., 1999a). Genome-wide studies by Sassetti et al. (2003) have shown that the predominant genes required for optimal growth of *M.tb* include a range of enzymes predicted to have essential metabolic functions. In this way, bioinformatic analysis has allowed for novel drug targets against the *M.tb* infection to be identified. Gene sequencing analysis has also become a popular tool in detecting drug resistance due to the increased accuracy that it provides over other drug resistance detecting technologies (Walzl et al., 2018). Next-generation sequencing (NGS) and whole genome analysis could potentially become a reference standard in identification of drug resistance genotypes (Walzl et al., 2018).

2.6.3. Molecular genetics and mutagenesis techniques

The development of molecular diagnostic technology testing for resistance to anti-TB drugs also continues to move forward rapidly. Of utmost urgency is the discovery and development of biomarkers detecting the progression from latent infection to clinical active disease (Walzl et al., 2018). Sophisticated bio-informatic computational tools together with serological studies and protein/gene expression studies are being applied alongside systems biology approaches in the discovery and validation of such biomarkers (Walzl et al., 2018). Such systems-level approaches enhance and complement standard classical molecular biology techniques to investigate pathogens leading to an in-depth understanding of their interactions with the human host (Banaei-Esfahani et al., 2017).

In evaluating the role played by proteins in persistence and virulence, knockout mutants generated by targeting genetic approaches allow for the extensive study of specific genes in both *in vitro* and *in vivo* model systems (Boël et al., 2005). This type of work is valuable in that specific attenuated or potentially non-pathogenic strains can be generated for use in the development of vaccines (Boël et al., 2005). The technique of qRT-PCR applied in mutant or essentiality studies effectively assesses specific *M.tb* gene regulation, with the expression patterns noted in tissue culture or *in vitro* models of infection allowing for the complex interactions between pathogen and human host to be elucidated (Waddell & Butcher, 2007). Gene expression data overlaid onto metabolic pathway analyses become increasingly informative in understanding organisms such as *M.tb*, by helping to define the physiological state of infecting

mycobacteria (Waddell & Butcher, 2007). A large amount of data can be gathered on the functional significance of target genes (in areas of importance such as mRNA degradation, protein expression, processing and regulation networks) using mutagenesis and knockout/down techniques (Waddell & Butcher, 2007).

The application of molecular genetics in the study of *M.tb* became a true reality upon application of methods transforming genomic DNA, such as transposon mutagenesis, directed allelic exchange, electroporation and phage-mediated specialized transduction methods for DNA transfer. Such methods enable the engineering of mutant strains by generating specific and defined mutations in *M.tb* (Braunstein et al., 2002).

These methods, although steadily being enhanced by further development, are faced with many of the same issues as other classical and molecular techniques. Progress is impeded by the common core difficulties faced when working with *M.tb*, including slow growth, the need for special biosafety precautions, as well as a tendency to aggregate and clump during growth which hinders the isolation of individual clones (Braunstein et al., 2002).

2.7. Significance of the study

The worldwide WHO End TB Strategy by 2035 targets a 95% reduction in TB mortality and a 90% reduction in TB incidence (Taghizade et al., 2016). In order for us to significantly move closer toward reaching the targets, the development of a dual-effect vaccine with prophylactic effects in uninfected adults needs to take precedence. Such a vaccine should be able to serve as an immunotherapeutic adjunct to drug therapy intended to reduce treatment duration in those with active TB by reducing bacterial loads.

The above review establishes and confirms a critical urgency in the need for discovery and validation of potential novel gene and protein targets, such as the adhesins HBHA and MTP, for drug and vaccine development, in an effort to eradicate TB. Thus, this study focused on establishing the effect of the combined removal of the two main adhesin proteins, to understand their roles independently and cumulatively on growth, viability and biofilm formation. In addition, the adhesion and invasion capacity of these molecules on epithelial cells, *in vitro* was elucidated.

2.8. Research design

2.8.1. Aim of study:

To establish the contribution of the known *M.tb* adhesin molecules, HBHA and MTP, in the pathogenesis of *M. tuberculosis*, by assessing changes in the host-pathogen interaction relative to the absence or presence of both genes in a double-knockout mutant strain of V9124.

2.8.2 Study objectives

1. Generate and confirm a single knock-out mutant of *hbhA* in the F15/LAM4/KZN susceptible strain V9124 by mycobacterial specialized transduction
2. Generate and confirm double mutant of *hbhA-mtp* in a confirmed *hbhA* mutant strain by mycobacterial specialized transduction
3. Complement the double mutant strain with both genes in plasmids independently, to confirm restoration of gene function
4. Perform growth and viability assays on double *hbhA-mtp* mutant strains concurrently with the wildtype and complemented strains (Optical Density A_{600}, Colony Forming Unit counts and Rezazurin Microplate Assay)
5. Evaluate host-pathogen interactions by assessing adherence to and invasion of A549 cell lines by the wildtype, single mutant, double mutant and complemented strains

2.8.3. Thesis overview

This thesis is comprised of 5 chapters:

- Chapter 1 – a general introduction and literature overview of *M.tb* and the current issues relating to the disease, with rationale for the focus on adhesins and their potential for use in drug and vaccine development.

- Chapter 2: **"*Mycobacterium tuberculosis adhesins*: potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets"** – a published literature review focusing on the various families of *M. tuberculosis* adhesin molecules with discussion regarding the current need for novel vaccine targets and drug candidates

- Chapter 3: **"*Mycobacterium tuberculosis* heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) are essential for *in vitro* growth, but not viability and biofilm production."** - original research paper discussing the rationale for targeting multiple adhesins for potential vaccine development, and elaborates on the technical deletion of two adhesin genes by specialized transduction followed by data generated from phenotypic assays.

- Chapter 4: **"The *Mycobacterium tuberculosis* HBHA and MTP proteins jointly support adhesion to and invasion of A549 pulmonary epithelial cells."** - original research paper discussing data generated from phenotypic assays utilizing the double mutant and complemented strains

- Chapter 5 - synthesis of the data chapters, demonstrating how they are inter-related, ending with the final overall conclusions, potential outcomes and recommendations for future perspective.

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The above chapter presented a general introductory overview of the core issues and presented a brief background of *Mycobacterium tuberculosis* and the current issues relating to the disease. The problem statement rationalized the need for the study and the study design, and outcomes were briefly discussed.

The next chapter is a published literature review focusing on the various *M. tuberculosis* adhesin molecules with discussion centered around some of the well-characterized and novel, putative *M. tuberculosis* adhesins. Additionally, the current need for novel vaccine targets and drug candidates, as well as the lucrative prospect of the use of adhesins as potential therapeutic targets is elaborated on.

CHAPTER 2: Paper 1 – "Mycobacterium tuberculosis adhesins: potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets"

Mycobacterium tuberculosis adhesins: potential biomarkers as anti-tuberculosis therapeutic and diagnostic targets

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1.1. Abstract

Adhesion to host cells is a precursor to host colonization and the evasion of the host immune response. Conversely, it triggers the induction of the immune response, a process vital to the host's defence against infection. Adhesins are microbial cell surface molecules or structures that mediate the attachment of the microbe to host cells and, thus, the host-pathogen interaction. They also play a crucial role in bacterial aggregation and biofilm formation. In this review, we discuss the role of adhesins in the pathogenesis of the etiological agent of tuberculosis, *Mycobacterium tuberculosis*. We also provide insight into the structure and characteristics of some of the characterized and putative *M. tuberculosis* adhesins. Finally, we examine the potential of adhesins as targets for the development of TB control strategies.

1.2. Introduction

M. tuberculosis, the etiological agent of tuberculosis (TB), continues to pose a challenge to global public health. Despite more than a century of research, this non-

discriminant pathogen continues to infect roughly a third of the world's population (WHO, 2013). Whilst there has been a significant reduction in TB cases and deaths in the past two decades, approximately 8.6 million new cases and 1.3 million people succumbed to the disease in 2012, despite the availability of cheap, efficacious, and curative therapy for TB (WHO, 2013). The synergistic relationship between HIV and *M. tuberculosis* infection, and the significant increase in the prevalence of multi-, extensively-, and totally-drug resistant (MDR, XDR, and TDR) *M. tuberculosis* strains (Gillespie, 2002; Fauci et al., 2008; Jassal & Bishai, 2009; LoBue, 2009; Velayati et al., 2009; Almeida Da Silva & Palomino, 2011) are largely accountable for the dramatic resurgence of TB as a serious global public health epidemic. This is further complicated by the lack of an effective vaccine (Russell et al., 2010), prolonged chemotherapy regimens (Mitchison & Davies, 2012), and adverse TB/HIV drug interactions (Luetkemeyer et al., 2011). Knowledge on the mechanisms utilized by *M. tuberculosis* to infect the host would offer novel perspective and define new targets to facilitate the design and development of drugs that are effective against both sensitive and resistant organisms (Ginsberg & Spigelman, 2007), efficacious vaccines (Bermudez et al., 2002), as well as crucially needed rapid, accurate, and cheap point-of-care tests (Wallis et al., 2010).

It is well established that adherence molecules (adhesins) play a fundamental role in the pathogen-host interaction (da Silva Neto et al., 2009; Espitia et al., 2012). Invasion of host cells by bacteria is a complex process involving both bacterial and host cell determinants (Bermudez & Goodman, 1996; Danelishvili et al., 2003). Pathogenic bacteria, such as *M. tuberculosis*, must initially adhere to and invade eukaryotic cells as a survival mechanism, enabling host colonization and the evasion of host immune defences (Niemann et al., 2004; Pizarro-Cerdá & Cossart, 2006; Kline et al., 2009). Adherence and invasion mechanisms have been well studied in pathogenic bacteria and fungi (Finlay & Cossart, 1997; Pizarro-Cerdá & Cossart, 2006; Singh et al., 2012a; Monack & Hultgren, 2013; Foster et al., 2014). These have illustrated that adhesins are the key players in the interactions that occur between the pathogen and the host, operating either as intercellular adhesion molecules (ICAM) or substrate adhesion molecules (SAM) (da Silva Neto et al., 2009). In this capacity, they are able to facilitate either cell-to-cell or cell-to-extracellular matrix (ECM) adherence and are usually surface-exposed (da Silva Neto et al., 2009). Adhesins also function in surface colonization and bacterial cell aggregation by facilitating cell-to-cell contact, leading

to the formation of microbial community structures or biofilms, a key contributor to microbial persistence (Barnhart & Chapman, 2006). Adhesins are, therefore, essential to microbial pathogenesis and are considered important biomarkers for diagnostics and therapeutics.

In this review, we focus on the role played by adhesins in TB pathogenesis; we discuss some of the major *M. tuberculosis* adhesins; and examine their potential as targets for the development of anti-TB strategies.

1.3. The host-pathogen interaction and adhesins

The initial interaction of the bacilli with the host immune system has been reported to occur in the lungs with the alveolar macrophage, the host cell within which *M. tuberculosis* replicates (Algood et al., 2003; Smith, 2003). Under optimal conditions, these immune cells engulf the bacterium into a phagosome, leading to its destruction (Dubnau & Smith, 2003). However, *M. tuberculosis* has evolved mechanisms that allow it to thrive within the harsh environment, by delaying phagosome maturation (Armstrong & Hart, 1971; Russel, 2001; Nguyen & Pieters, 2005). If this fails, the bacteria are able to escape death by entering the cytosol (van der Wel et al., 2007). TB-infected macrophages can travel to the hilar lymph nodes and bloodstream (Henderson et al., 1963; Harmsen et al., 1985) and this interaction is critical in the dissemination and systematic spread of the pathogen.

Although the primary mechanism of infection is reported to be via macrophages, epithelial cells are present in far larger numbers than macrophages within alveoli (Crandall & Kim, 1991). The first cells that *M. tuberculosis* encounter in the lung are, therefore, most likely to be epithelial cells. *M. tuberculosis* is versatile in that it is capable of infecting and growing in these pneumocytes *ex vivo* (Bermudez & Goodman, 1996; Mehta et al., 1996; Ashiru et al., 2010). The *M. tuberculosis*-epithelial cell interaction may potentially precede invasion of the macrophage and could also then facilitate the recruitment of macrophages to the site of infection by chemokine secretion (Alteri, 2005). Epithelial cells also present a niche within which the bacilli are afforded direct access to the host lymphatic and blood systems without the need for carrier macrophages. This is affected by the disruption and destruction of the alveolar vascular endothelium due to the cytotoxicity of *M. tuberculosis* to human pneumocytes (McDonough & Kress, 1995; Dobos et al., 2000; Castro-Garza et al., 2002).

Dendritic cells are better antigen presenters than macrophages and are key players in the early stages of TB infection (Tascon et al., 2000) and activate T cells with specific *M. tuberculosis* antigens (Bodnar et al., 2001; Gonzalez-Juarrero & Orme, 2001). Dendritic cells, however, do not support intracellular growth of *M. tuberculosis* but maintain the live bacteria in vacuoles (Tailleux et al., 2003). Since dendritic cells are migratory, they may potentially facilitate the dissemination of *M. tuberculosis* in this way (Lipscomb & Masten, 2002).

Microbial adhesins are crucial to bacterial attachment to host cells (Fig. 1.1). The interaction of bacterial adhesins with host cell receptors is a key determinant of host specificity and tissue tropism (Klemm & Schembri, 2000). Furthermore, the cell surface location of adhesins and their role in attachment to host cells leads to the triggering of immune responses, which are crucial to the host's defence against infection (Barnhart & Chapman, 2006; Bergsten et al., 2007).

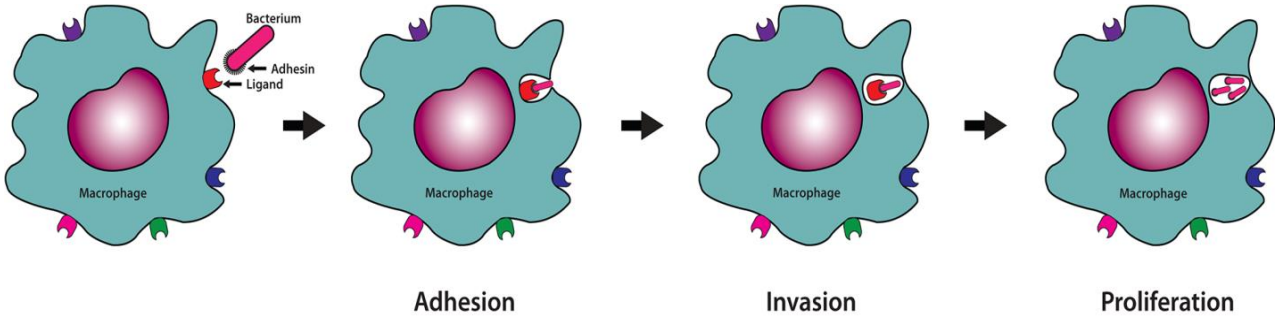

Fig. 1.1. The role of adhesins in the infection of host cells. Adhesins on the bacterium surface bind to molecules (ligands) on the host cell (in this instance, a macrophage) that are usually not meant for this purpose. This leads to invasion of the bacterium and its subsequent ability to survive and replicate within the host cell.

1.4. Bacterial adhesins and their mechanisms of interaction with the host

Adhesins serve to fulfil a common basic role, which is to initiate close contact with a receptor-like domain on the host cell surface, forming a fundamental link between the host and bacterium (Gerlach & Hensel, 2007). Infectious organisms have evolved to express novel adhesins that are able to bind to molecules ordinarily located on the eukaryotic cell surface. Molecules such as integrins, glycosaminoglycans, and specific sugar residues have all been shown to interact with adhesins of pathogens (Delogu & Brennan, 1999; Dersch & Isberg, 2000; Brennan et al., 2001). Most bacteria express a number of variable adhesins on their surfaces that have specific affinity to terminal

sugar residues or internal sequences in oligosaccharide chains that help to define the microbe's ecological niche (Esko & Sharon, 2009). A range of adhesins have been described and characterized over the years. Lectin adhesins function predominantly to interact with glycan ligand receptors on the surface of host cells (Esko & Sharon, 2009). In addition, two major classes of protein adhesins have been defined to date: the fimbrial and the non-fimbrial adhesins (Gerlach & Hensel, 2007). Fimbriae (hairs) or pili (threads) are the most common form of bacterial adhesins. They are elongated, multi-subunit protein structures that are able to interact with glycoprotein and glycolipid receptors found on host cells (Esko & Sharon, 2009).

In order to be functional, adhesins must be anchored onto or displayed on the bacterial cell surface with their functional domain on display (Chhatwal, 2002). Bacteria have developed several anchoring mechanisms involving the presence of a signal peptide or unique motifs (Chhatwal, 2002). The majority of adhesins function by binding ECM components, such as fibronectin. This is often considered to be an essential binding molecule facilitating bacterial adherence, due largely to its ability to bind both host cells and bacteria (Henderson et al., 2011). Fibronectin is also regarded as an effector in the triggering of signal transduction events that result in bacterial invasion of eukaryotic cells through interaction with integrins (Joh et al., 1999; Chhatwal, 2002). *M. tuberculosis*, specifically, has been shown to express adhesins that are capable of binding to ECM proteins, such as proteoglycans and fibronectin (Brennan et al., 2001).

Proteins commonly secreted by bacterial pathogens can, in some instances, be 'anchorless adhesins' which facilitate colonization of host organisms (Gerlach & Hensel, 2007). Following their secretion, they accumulate and re-associate with the bacterial surface where they are able to execute biological functions, including host adherence and entry (Bergmann et al., 2001; Chhatwal, 2002).

1.5. Adhesins central to *M. tuberculosis* pathogenesis

Several studies have identified multiple *M. tuberculosis* proteins capable of interacting with receptors on host cells to facilitate binding to mammalian components and these are classified as mycobacterial adhesins (Fig. 1.2) (Menozzi et al., 2006; Kumar et al., 2013). Pethe et al. (2002) identified a laminin binding protein involved in cyto-adherence by its recognition of laminin. Kinhikar et al. (2006) showed that the glyoxalate pathway enzyme, malate synthase (*glcB*; Rv1837c), binds to the human

ECM proteins laminin and fibronectin and is an anchorless adhesin. The 19-kDa lipoprotein antigen (Rv3763) present on the cell wall preferentially binds to THP-1 macrophage-like cells (Diaz-Silvestre et al., 2005). The cell surface glycoprotein Apa (Rv1860), initially considered a secreted molecule, has been shown to transiently associate with the cell wall in order to allow attachment to the pulmonary surfactant protein-A (PSP-A) (Ragas et al., 2007). The Cpn60.2 molecular chaperone protein (*GroEL2*; Rv0440), believed to be involved in bacterial pathogenicity and considered essential for cell viability, appears to be necessary to facilitate efficient bacterial association with macrophages (Stokes et al., 2004). Recently, Kumar et al. (2013) identified Rv2599 (membrane protein), Rv0309 (L,D-transpeptidase), and Rv3717 (N-acetylmuramoyl-L-alanine amidase) as novel adhesins of *M. tuberculosis* H37Rv, capable of binding to laminin and fibronectin.

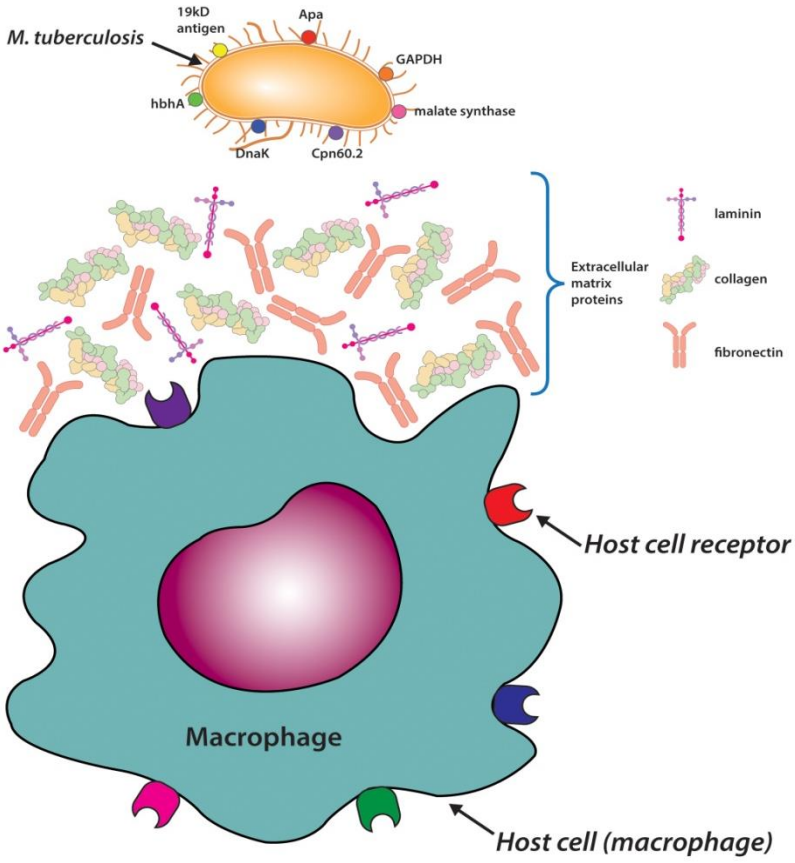

Fig. 1.2. Selected *M. tuberculosis* adhesins and the multiple pathways available for initiating host interaction and colonization (Modified from: Kumar et al., 2013).

1.5.1. Heparin-binding hemagglutinin adhesin (*hbhA*; Rv0475)

The most characterized and major adhesin in *M. tuberculosis* is the 28-kDa heparin-binding hemagglutinin adhesin (HBHA). This surface-exposed protein is a virulence factor that facilitates the dissemination of *M. tuberculosis* from the site of primary infection by initiating interaction with host epithelial cells (Menozzi et al., 1996; Menozzi et al., 1998; Pethe et al., 2001; Menozzi et al., 2006; Esposito et al., 2011). The two crucial steps in TB pathogenesis, namely bacterial aggregation and cell adhesion, are facilitated by HBHA (Menozzi et al., 1998; Esposito et al., 2012). HBHA-mediated aggregation is instrumental in the formation of bacterial clumps, allowing for more effective adherence and invasion (Esposito et al., 2012; Lebrun et al., 2012). A definitive role for HBHA in the facilitation of host adherence was shown by Menozzi et al. (1996) in which antibodies directed against HBHA inhibited attachment of mycobacteria to epithelial cells. In support of this, patients with active TB have been shown to produce anti-HBHA antibodies, suggesting HBHA expression during human infection (Menozzi et al., 1998). The carboxy-terminal lysine-rich domain of HBHA functions in recognizing heparan sulphate-containing receptors on epithelial cells (Delogu & Brennan, 1999; Pethe et al., 2000). Pethe et al. (2001) showed that colonization of a *M. tuberculosis hbhA* mutant strain in the lungs of mice was equivalent to that of the wild-type strain. However, the mutant displayed a reduced capacity to disseminate from the lungs to other regions of the body, suggesting the role of HBHA in extrapulmonary spread. The authors also showed that an antibody against the carboxyl-terminal domain of HBHA blocks binding to epithelial cell receptors, impeding extrapulmonary spread of *M. tuberculosis* in the mouse model (Pethe et al., 2001). This suggests that the humoral immune response to HBHA, and possibly other *M. tuberculosis* adhesins, could potentially play a protective role in blocking dissemination from the lungs (Alteri, 2005).

1.5.2. *M. tuberculosis* pili

Pili proteins are hydrophobic adhesion molecules used by a wide range of bacterial pathogens to infect host cells (Finlay & Falkow, 1997). Structurally, pili are generally composed of pilin subunits with an adhesin tip, fashioned into straight or flexible filaments 1-10 nm wide and 0.07-3 µm long (Telford et al., 2006). Several virulence-associated functions, including agglutination of human and animal erythrocytes, bacterial adherence/aggregation, biofilm formation, and adherence and colonization of

mucosal surfaces can be functionally attributed to pili (Strom & Lory, 1993; Finlay & Falkow, 1997). The hydrophobicity of the pilin adhesin enables interaction between bacteria and eukaryotic cells (Klemm & Schembri, 2000). Several distinct pilus types have been identified, the most characterized of which are the type I pili (produced by enteropathogenic *Escherichia coli*), type IV pili (produced by *E. coli* and *Pseudomonas* and *Neisseria* species), and curli pili (produced by some strains of *E. coli*) (Telford et al., 2006). The previous misconception that mycobacteria are not piliated was clarified by Alteri (2005), who proved, using transmission electron microscopy of negatively-stained bacilli, that *M. tuberculosis* produces two physically-distinct pili morphotypes: type IV and curli-like pili (MTP).

The type IVB pilus locus of *M. tuberculosis* encodes a prepilin of the Flp pili family (Alteri, 2005). In addition, the proteins encoded by the Rv0990c and Rv2551c ORFs are thought to be involved in the secretion or cleavage of the Flp prepilin substrate (Alteri, 2005). Genetic analysis revealed that *M. tuberculosis* acquired these genes by horizontal gene transfer as the *flp* genes were shown to be flanked by multiple direct repeats, suggesting insertion of foreign DNA into its chromosome (Alteri, 2005). The role of this pilus type as an adhesin in this organism has yet to be determined. In Gram-negative organisms, type IV pili function in adherence to host tissue, co-aggregation, immunomodulation, motility, and DNA uptake (Telford et al., 2006).

Alteri et al. (2007) showed that purified MTP are comprised of 4-kDa protein subunits, encoded by the Rv3312A ORF. These researchers also demonstrated that MTP are produced during pathogenesis and play a role in stimulating the humoral immune response. This was evidenced by the sera of patients with active TB containing IgG antibodies against MTP (Alteri et al., 2007). Furthermore, MTP binds to laminin *in vitro* and are produced during adherence to epithelial cells, implying that they serve as an adherence factor that is crucial in mediating close interaction and colonization with host cells (Alteri et al., 2007). Their role as an adherence factor was further substantiated by their involvement in cellular aggregation and biofilm formation (Ramsugit et al., 2013) and in the adhesion to and invasion of THP-1 macrophages (Ramsugit & Pillay, 2014).

1.5.3. Apa (alanine-proline-rich antigen; Rv1860)

The 45-47-kDa secretory and cell surface antigen Apa (alanine-proline-rich antigen; Rv1860) is a mycobacterial glycoprotein whose expression appears to be restricted to

the members of the *M. tuberculosis* complex, including the vaccine strain *M. bovis* Bacille Calmette-Guérin (BCG) (Ragas et al., 2007; Nandakumar et al., 2013). The secreted antigen is not present in other mycobacterial species, including *M. avium, M. marinum,* or *M. smegmatis* (Ragas et al., 2007; Nandakumar et al., 2013). Apa is targeted by, and binds directly to the human PSP-A, an innate immune system C-type lectin responsible for early recognition of invading pathogens (Ragas et al., 2007). The secreted antigen remains associated with the cell wall only long enough to facilitate its attachment to PSP-A. Ragas et al. (2007) demonstrated the presence of a structural determinant in the manno-oligosaccharide moiety that has been highly implicated in the adhesion function of the protein. They also confirmed the adhesion function of Apa during TB infection and pathogenesis. The immune-dominant Apa possesses fibronectin-binding activity and is strongly recognized by serum antibodies of active TB patients. In addition, it shares significant amino acid homology with a fibronectin attachment protein family common to other mycobacterial species, including *M. avium, M. marinum,* and *M. leprae* (Nandakumar et al., 2013). In *M. tuberculosis* specifically, mannosylated Apa plays a key role in host cell interaction (Nandakumar et al., 2013). Apa has been proposed as a possible vaccine candidate or component for future vaccines against TB. This has been supported by Nandakumar et al. (2013) who showed Apa to offer significant protection against virulent *M. tuberculosis* in mice when used as a BCG-booster vaccine.

1.5.4. Malate synthase (*glcB*; Rv1837c)

The glyoxalate shunt, a key pathway in fatty acid metabolism of *M. tuberculosis* during persistent infection, has long been hypothesized to be a weakness in the bacteria's armour that could potentially be exploited for the development of anti-tubercular therapeutics (Krieger et al., 2012). Da Silva Neto et al. (2009) showed that malate synthase, an enzyme of the glyoxalate pathway, from *Paracoccidioides brasiliensis* is a multifunctional protein, with a dual role as an enzyme and mediates adherence of the fungus to host cells via its ability to bind fibronectin and type I/type IV collagen. Malate synthase has been suggested to be actively secreted by this fungal pathogen in a similar manner to *M. tuberculosis* (da Silva Neto et al., 2009). In *M. tuberculosis*, the single malate synthase encoded by *glcB* is thought to play a key role in pathogenesis by imparting some degree of virulence to the bacteria (Dunn et al., 2009). Kinhikar et al. (2006) have demonstrated *in vivo* expression of malate synthase to be typical during

active infection, with the enzymatic function suggesting a general cytoplasmic localization of the protein. However, this protein was also consistently identified as a secreted protein in the growth medium filtrates of mid-log phase cultures (Kinhikar et al., 2006). Studies have shown that secreted malate synthase enhances the adherence of the pathogen to lung epithelial cells by binding to the glycoproteins, laminin and fibronectin (Kinhikar et al., 2006; Dunn et al., 2009). The binding activity of malate synthase was shown to be mediated by the presence of a unique C-terminal domain. These features, together with the marked absence of a conventional secretion mechanism or defined cell wall anchoring motif, resulted in the malate synthase of *M. tuberculosis* being classified as an anchorless adhesin that is able to contribute to bacterial virulence by facilitating infection of the host and dissemination (Kinhikar et al., 2006).

1.5.5. Glyceraldehyde-3-phosphate dehydrogenase (*gap*; Rv1436)

The GAPDH protein family is known to have diverse functional activity depending on the sub-cellular location, secondary to its primary role in glycolysis and central carbon metabolism (Barbosa et al., 2006). Several authors have indicated that GAPDH may be either secreted or expressed on the cell surface in both Gram-positive and Gram-negative bacteria, parasites, and fungi (Pancholi, 2001; Pancholi & Chhatwal, 2003; Matta et al., 2010; Jin et al., 2011). In support of this, the GAPDH of group A streptococci has been shown to be membrane-bound and is able to bind fibronectin, lysozyme, and myosin and actin cytoskeletal proteins, pointing toward an additional function leading to bacterial colonization (Barbosa et al., 2006). The unusual extracellular localization of GAPDH has been confirmed in a broad range of other microorganisms, including *Staphylococcus* spp., *Neisseria meningitidis*, *E. coli*, *P. brasiliensis*, *Trichomonas vaginalis*, and *Candida albicans* (Dumke et al., 2011).

The surface-localized equivalent proteins have also been shown to remain enzymatically active and are transcribed from the same ORF as the cytoplasmic molecules (Purves et al., 2010). In its role as a surface-associated protein, GAPDH is able to interact with both specified host factors like fibronectin, fibrinogen, albumin, laminin, collagen, and plasminogen, as well as human epithelial and endothelial cells and fimbriae of other bacterial species (Dumke et al., 2011). GAPDH, specifically, has been shown to be important in the pathogenesis of *Staphylococcus aureus* infections (Purves et al., 2010). Tunio et al. (2010) showed a *N. meningitidis gapA* mutant to

adhere significantly less to human cells when compared to wild-type strains. Additionally, Boël et al. (2005) indicated that inhibiting export of GAPDH to the surface of group A streptococci affected its virulence by reducing adherence to target cells.

Despite the existence of a large body of literature on bacterial cell surface GAPDH, only two studies have shown direct evidence for the role of GAPDH as a cell surface protein that plays a role in bacterial virulence (Henderson & Martin, 2011). This is most likely attributed to the essentiality of the gene in its primary function in glycolysis, resulting in the inability to inactivate the encoding gene (Henderson & Martin, 2011). It is, thus, not surprising that a cell surface GAPDH knockout has, to date, not been evaluated *in vivo*.

1.5.6. Chaperonins as adhesins (*dnaK* and *groEL2*; Rv0350 and Rv0440)

Although a number of heat shock protein classes have been identified, GroESL and DnaK are regarded as the major response systems within bacteria (Singh et al., 2012b). The molecular chaperones constitute a diverse set of conserved proteins in bacteria, encoded by the essential heat shock protein genes *groEL* and *groES*. These genes, also called *cpn60* and *cpn10*, mediate the correct assembly, folding, transport, and degradation of other proteins *in vivo* (Qamra et al., 2004; Shahar et al., 2011; Singh et al., 2012b). *M. tuberculosis* contains two copies of the *cpn60* genes, with one of these genes, *cpn60.1*, organized in an operon with *cpn10*, while the second, *cpn60.2*, is arranged separately in the genome and is expressed from the *myc28* gene (Qamra et al., 2004; Lewthwaite et al., 2007; Shahar et al., 2011).

Recent mycobacterial studies demonstrated the unusual presence of the Cpn60s on the outside surface (capsule) of the bacterial cell. This is despite the predominant role of these proteins in the cytoplasm and the lack of a secretion signal sequence or other known motif implicating its export (Qamra et al., 2004; Shahar et al., 2011; Zhu et al., 2013). Hickey et al. (2009) have shown both the molecular chaperones Cpn60.2 and DnaK to be surface components of the mycobacterial capsule in cultured bacilli. The authors also showed Cpn60.2 to bind to the surface of macrophages at sites required for efficient association with *M. tuberculosis* bacilli, and that blocking surface-localized Cpn60.2 with antibodies resulted in a reduction of bacterial binding. Additionally, Hickey et al. (2009) showed that the Cpn60.2 protein is a major mycobacterial adhesin, which is able to stabilize the interaction between the pathogen and the alveolar

macrophage through CD43. Such studies implicate Cpn60.2 as having a secondary function as an adhesin molecule (Hickey et al., 2010; Shahar et al., 2011). They provided further evidence that mycobacterial molecular chaperone/cell stress molecules are vital moonlighting proteins, which are able to promote bacterial survival and virulence due to their irregular position outside of the cell (Henderson et al., 2010).

It was hypothesized that the secondary, extracellular functionality of these molecular chaperones may allow for synergism with the intracellular cell stress response in order to generate a novel homeostatic network of interactions (Henderson et al., 2010). Although the Cpn60.2 protein is expressed from outside the GroEL/ES-like operon, studies have shown the protein to be essential for survival, with deletion of the genes encoding Cpn60.2 completely preventing the growth of *M. tuberculosis* (Shahar et al., 2011). Hu et al. (2008) demonstrated an inability to produce *M. tuberculosis* mutants lacking the *cpn60.2* gene through recombineering, further suggesting the essentiality of this gene. Hickey et al. (2009) have shown that although both Cpn60.2 and DnaK proteins are present on the bacterial surface, only the former seems to be required to facilitate efficient bacterial interactions with macrophages.

1.6. Adhesins as targets for the development of TB control strategies

Significant further research into identifying and characterizing *M. tuberculosis* adhesins is required in order to elucidate their precise role in virulence, establish the mechanisms behind their secretion, and to identify the receptors required for their association with the host cell surface. Uncovering the mechanisms of binding of adhesins to host cells will, therefore, lead to an improved understanding of the initial events that occur during TB infection. Such studies could potentially result in the development of novel strategies for infection control by means of therapeutics that are able to block secretion and/or prevent re-association with the cell membrane. Secreted proteins also have potential as biomarkers for diagnostic use, as such molecules are recognized with sensitivity during the humoral response to infection by *M. tuberculosis*.

1.6.1. Development of anti-adhesives

Greater understanding of the adhesin-ligand interaction may facilitate the design of competitive inhibitors that block their formation, as has been shown in several other organisms, including *E. coli*. The conjugation of the QFGGN amyloid motif from the *E. coli* CsgA curlin to proline residues was shown to inhibit bacterial curli formation

(Cherny et al., 2005). Bicyclic 2-pyridones, or pilicides, target the chaperone-usher interaction, blocking pilus biogenesis, and led to an inhibition of adhesion to bladder cells and biofilm formation in *E. coli* (Pinkner et al., 2006). A multivalent galabiose derivative is an inhibitor of adherence by *E. coli* P-fimbriae (Salminen et al., 2007). Similarly, α-D-mannose based inhibitors targeting the FimH adhesin prevent *E. coli* adhesion on uro-epithelial cells, invasion, and biofilm formation (Wellens et al., 2008).

1.6.2. Adhesins as vaccine candidates

The importance of adhesins in pathogenesis implies that disrupting bacterial attachment by specific anti-adhesin antibodies has the potential to incapacitate a pathogen (Klemm & Schembri, 2000). However, the large number of different adhesin genes in an organism's genome suggests that there are multiple pathways for bacterial adherence (Brzuszkiewicz et al., 2006). This redundancy limits the use of adhesins as vaccine candidates (Kline et al., 2009). In addition, different adhesins are not produced simultaneously and continuously and require different environmental cues for expression and many exist as antigenic variants (Klemm & Schembri, 2000).

Even with these constraints, adhesins in several species have proven to be useful vaccine candidates, capable of conferring protection in animal models. Their extracellular location is also a favourable feature in that it enables their interaction with protective antibodies (Telford et al., 2006). Their multiple functions in pathogenesis and modulation of the host immune response, together with independent signalling events mediated by individual components of polymeric adhesins, also produces a more robust immune response (Mandlik et al., 2008).

A review by Kline et al. (2009) highlighted adhesive proteins as protective antigens. These include (amongst others): an *E. coli* FimH adhesin-based vaccine against cystitis in a primate model (Langermann et al., 2000), the *E. coli* Dr fimbrial antigen against urinary tract infection in mice (Goluszko et al., 2005), the *Salmonella* atypical fimbriae B chaperone (SafB) complexed with the SafD adhesin against invasive *Salmonella enteritidis* infection (Strindelius et al., 2004), a synthetic-peptide consensus-sequence vaccine (Cs1) against type IV pilus of *Pseudomonas aeruginosa* in a mice model (Kao et al., 2007), and a combination of three group B *Streptococcus* (GBS) pilus variants that mediate protection in mice against all tested GBS challenge strains (Margarit et al., 2009).

1.6.3. Adhesins as serodiagnostic markers for *M. tuberculosis*

Exposure to *M. tuberculosis* antigens leads to the production of specific antibodies, which may be used as markers of infection in serological tests. Several *M. tuberculosis* antigenic proteins have been evaluated as serological markers, particularly culture filtrate proteins, however, surface-exposed proteins appear to be more effective (Abebe et al., 2007).

The 19-kDa lipoprotein adhesin is recognized by sera from TB patients and the sensitivity of this immuno-dominant antigen was found to be 62% in sputum smear-negative TB patients (Jackett et al., 1988; Bothamley et al., 1992a, b; Greenaway et al., 2005). The anchorless adhesin malate synthase is a surrogate marker for TB infection in HIV-seropositive individuals (Abebe et al., 2007), with 57% of TB-infected and 92% of TB-HIV co-infected patients in Uganda and South Africa, respectively, showing reactivity to this antigen (Hendrickson et al., 2000). The 30-kDa antigen (antigen 85B or α-antigen) binds to fibronectin and is regarded as a potential *M. tuberculosis* adhesin (Ratliff et al., 1988). The sensitivity of this antigen, however, was shown to have a broad range of variability, from 41-94% (Vikerfors et al., 1993; Lim et al., 1999; Raja et al., 2002; Uma Devi et al., 2003; Raja et al., 2004). Delogu & Brennan (2001) showed that mice infected with *M. tuberculosis* by aerosolization generated antibodies against the PE_PGRS protein Rv1818. This protein has been suggested to be an adhesin that is involved in both the infection of macrophages and in cell aggregation (Brennan et al., 2001).

HBHA may also be a useful target in TB diagnostics due to its presence in the early stages of TB infection (Masungi et al., 2002). Zanetti et al. (2005) reported that 80% of TB patients (n = 5) presented with increasing HBHA antibody titres over a four-month period. Two of these patients showed a high HBHA antibody level, even though they were tuberculin skin test negative. With regard to the onset of the immune response, methylation of HBHA appears to be crucial for the induction of T cell antigenicity and protective immunity against *M. tuberculosis* (Temmerman et al., 2004). Methylated HBHA may, thus, be a suitable diagnostic marker for identifying individuals with active TB (Abebe et al., 2007).

It was recently shown that the *mtp* gene, encoding curli-like pili (MTP), is a conserved gene present in *M. tuberculosis* complex strains, but not in non-tuberculous mycobacteria or other respiratory bacteria (Naidoo et al., 2014). In addition, sera of patients with active TB are known to contain IgG antibodies against MTP (Alteri et al.,

2007). MTP may, therefore, be a suitable marker for the development of a point-of-care TB test.

1.7. Conclusions

The involvement of *M. tuberculosis* surface molecules in adherence is essential to the increased understanding of the bacterium's pathogenesis. However, little progress has been made in identifying and characterizing the contribution made by the specific adhesins utilized by this pathogen during infection of host cells. This can be attributed to a plethora of factors that include technical difficulties in generating gene knock outs, an inability to knock out genes of essential function, a lack of studies on *M. tuberculosis* entry into non-phagocytic cells, the previous mistaken notion that *M. tuberculosis* is non-fimbriated, and the under-appreciation of the pellicle biofilm lifestyle of this pathogen. Further work on identifying novel adhesins and defining their contribution to TB pathogenesis is critical to increasing our understanding of the physiology and molecular mechanisms of TB pathogenesis that may subsequently facilitate the development of new strategies for TB control.

1.8. References

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The above paper presented a detailed a review that discussed the key function and importance of adhesins in the pathogenesis of the aetiological agent of tuberculosis, *Mycobacterium tuberculosis*. The review provides details regarding the features of both well-characterized and putative novel *M.tb* adhesins. The review provides rationale and advocates for the potential of adhesins as targets for the development of therapeutic strategies.

In proof of this concept, the next chapter assesses the potential importance of HBHA and MTP as a combined target for therapeutic drug and vaccine development by establishing the *in vitro* growth, viability and biofilm formation capacity.

CHAPTER 3: Paper 2 – "Mycobacterium tuberculosis heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) are essential for in vitro growth, but not viability and biofilm production."

Title: *Mycobacterium tuberculosis* heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) are essential for *in vitro* growth, but not viability and biofilm production.

Running title: HBHA and MTP are essential for *M. tuberculosis* growth, but not viability and biomass

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Abbreviations: *hbhA*/HBHA (heparin binding haemagglutinin adhesin), *mtp*/MTP (*Mycobacterium tuberculosis* pili), *M.tb* (*Mycobacterium tuberculosis*), TB (tuberculosis), KZN (KwaZulu-Natal), LB (Luria-Bertani), OADC (oleic acid, albumin dextrose and catalase), ADS (Albumin-Dextrose-Saline), DNA (deoxyribonucleic acid), CDS (coding sequence), ORF (open reading frame), MP (mycobacteriophage), PCR (polymerase chain reaction), MCS (multiple cloning site), TBE (Tris-Borate-EDTA), SAP (shrimp alkaline phosphatase), OD (optical density) PBS-T (phosphate buffered saline-Tween-80), CFU (colony forming units), REMA (resazurin microtiter assay), AES (allelic exchange substrate), ΔDM (double mutant), gDNA (high molecular weight genomic DNA)

Abstract: It is well established that the adhesion and invasion capacity of a bacterial pathogen is a major determining factor and precursor to effective host colonization and the onset of infection of a host. Bacterial adhesins are molecules present on the pathogen surface that facilitate attachment of the invading organism to the infected organism, and are thus vital in the host-pathogen interaction. In this study, the dual contribution of two of these vital adhesin proteins, HBHA (heparin binding haemagglutinin adhesin) and MTP (*Mycobacterium tuberculosis* curli pili) and their dual contribution to the growth, viability and biofilm production was investigated in *M.tuberculosis* (*M.tb*). A double mutant strain, Δ*hbhA-mtp*, deficient in the *hbhA* (heparin binding haemagglutinin adhesin) and *mtp* (*Mycobacterium tuberculosis* pili) genes was generated by phage-mediated mycobacterial specialized transduction. The double mutant strain was used to construct independent *hbhA*- and *mtp*- complemented strains. *In vitro* assays investigated differences in growth, viability and biofilm formation between the *M.tb* double mutant, complemented and wildtype F15/LAM4/KZN drug-sensitive strains. Differences in growth were measured by colony forming unit (CFU) counts and OD_{600nm} readings, whilst viability was determined by the resazurin microplate assay. Biofilm formation was assessed by means of crystal violet staining to quantify cellular biomass. The findings demonstrate the significance of these two major adhesins in facilitating growth and biomass formation (to a less significant degree), but no effect on the metabolic viability of *M.tb*. The results indicate the potential of the two major adhesins, HBHA and MTP, when combined as biomarkers, or used as targets for novel drug and vaccine development.

Introduction:

Approximately 1.67 million lives are lost to tuberculosis (TB) globally each year despite the availability of cheap, efficacious, and curative therapy (Bermudez et al., 2002; Algood et al., 2003; Almedia et al., 2008; Chang et al., 2007, World Health Organization, 2017). A better understanding of the mechanisms utilized by *Mycobacterium tuberculosis* (*M.tb*) to infect the host may offer novel perspectives and knowledge for the potential development of effective therapeutic measures and an innovative, efficacious vaccine (Bermudez et al., 2002; Boggiano et al., 2017).

Adhesion of bacilli to the host surface during infection is a crucial prerequisite in the pathogenic process, allowing for host colonization (Barbosa et al., 2006). Pathogenic bacteria adhere to and invade eukaryotic cells primarily to evade the immunological defense mechanisms mounted by the host as a means of survival, resulting in disease progression in the host (Cambier et al. 2014). Adherence molecules play a fundamental role in the pathogen-host interaction (da Silva Neto et al., 2009). Additionally, bacteria express numerous surface structures in order to locate their appropriate host target cells (Brennan et al., 2001).

Specific cell-surface molecules termed adhesins, initiate close contact between a receptor-like domain and a ligand on the host cell surface (Gerlach & Hensel, 2007) and mediate bacterial adherence to host cells, facilitating both invasion of and colonization within the host (Chhatwal, 2002). Mycobacteria display multiple proteins with primary adhesin function that interact with receptors on host cells (Menozzi et al., 2006).

Fimbriae (hairs) or pili (threads) are the most commonly found form of bacterial adhesins, and are able to interact with glycoprotein and glycolipid receptors found on host cells (Esko & Sharon, 2009). Pili proteins are key hydrophobic adhesion molecules used by bacterial pathogens as a mechanism to initiate infection of host cells (Finlay & Falkow, 1997) with the adhesin tip aiding in their structural, virulence-associated functions (Telford et al., 2006). These include bacterial adherence and aggregation, colonization of mucosal surfaces and agglutination of erythrocytes (Strom & Lory, 1993; Finlay & Falkow, 1997). *M.tb* produces two physically distinct pili morphotypes: curli-like pili (MTP) and type IV pili (Alteri, 2005). MTP proteins are produced during

pathogenesis (in adherence to epithelial cells), and play a key role in stimulating the humoral immune response (Alteri et al., 2007). Their role as an adherence factor was further confirmed by Ramsugit et al. (2013) who highlighted their involvement in cellular aggregation and biofilm formation, as well as in the adhesion to, invasion of, and survival in THP-1 macrophages and A549 pulmonary epithelial cells (Ramsugit & Pillay, 2014; Ramsugit et al., 2016; Ramsugit & Pillay, 2019). Interestingly, MTP has been noted to have only a limited effect on cytokine modulation in epithelial cells (Ramsugit et al., 2016). Naidoo & Pillay (2017) extensively reviewed the evidence justifying the potential value of this major adhesin as a focus for the design of rapid TB diagnostics for use in low-income settings. The MTP antigen was identified as a suitable biomarker for a TB diagnostic test by Naidoo et al. (2018) whereby anti-MTP antibodies were shown to be present in the serum of HIV positive and negative patients with active TB.

The 28-kDa heparin-binding haemagglutinin adhesin (HBHA), a major adhesin of *M.tb*, is a virulence factor reported to facilitate the dissemination of *M.tb* from the site of primary infection by initiating interaction with epithelial cells (Menozzi et al., 2006; Esposito et al., 2011). HBHA mutant strains showed a reduced ability to bind epithelial cells and disseminate from the lungs to other tissues (Menozzi et al., 2006; Esposito et al., 2011). The protein was demonstrated to be expressed on the bacterial surface and responsible for adhesion to epithelial cells and bacterial aggregation (Esposito et al., 2011, Esposito et al., 2012). HBHA-mediated aggregation is instrumental in the formation of bacterial clumps, allowing for more effective adherence and invasion (Esposito et al., 2012).

M.tb has been demonstrated to successfully adhere to, invade, and replicate within pulmonary epithelial cells (Bermudez & Goodman, 1996; Ashiru et al., 2010), a potentially safe environment for infecting bacilli to replicate away from the bactericidal mechanisms mounted by macrophages (Castro-Garza et al., 2002). Epithelial cells also present a niche within which the bacilli are afforded direct access to the host lymphatic and blood systems (effected by a disruption and destruction of the alveolar vascular endothelium), without the need for carrier macrophages (Castro-Garza et al., 2002).

Given the increasing evidence supporting the role of MTP and HBHA in *M.tb*

pathogenesis, these two major adhesins in combination may potentially be a significant target for the design of novel, effective drugs and vaccines. In the current study, a *M.tb* mutant strain deficient in both adhesin genes, Rv3312A (*mtp*) and Rv0475 (*hbhA*), was constructed. The combined contribution of MTP and HBHA on growth kinetics, viability and biofilm production was evaluated in wildtype and double knockout mutant strains. Restoration of gene function in the mutant was assessed by the generation of complemented strains.

Methods:

1. Bacterial strains and growth conditions

Bacterial strains used in the study are described in Table 1. The Dh5α *Escherichia coli* (*E. coli*) stocks containing the *hbhA* Allelic Exchange Substrate (AES) phasmids were grown in Luria-Bertani (LB) broth or on LB agar (Difco) for transformation, plasmid isolation and isolation of recombinant clones. *M. smegmatis* mc^2155 was grown in 7H9 (Difco) medium supplemented with 10% ADS (Albumin-Dextrose-Saline), 0.5 % (v/v) glycerol (Sigma, St. Louis, MO, USA) and 0.05% Tween-80. Mycobacterial strains other than *M. smegmatis* were grown in 7H9 (Difco) medium supplemented with 10% OADC (oleic acid, albumin dextrose and catalase), 0.5 % (v/v) glycerol (Sigma, St. Louis, MO, USA) and 0.05% Tween-80, or 7H10/ 7H11 (Difco) medium supplemented with 10% OADC. The *E.coli* stocks containing the *hbhA* and *mtp* Allelic Exchange Substrate (AES) phagemids used for specialized transduction were received from collaborators at the W.R. Jacobs laboratory at the Albert Einstein College of Medicine, the Bronx, USA.

When required for selective growth, antibiotics were used at the following concentrations: kanamycin at 20 µg/mL for *M.tb* complemented strains, and 50 µg/mL for *E. coli*; hygromycinB at 150 µg/mL for *E. coli*, 50 µg/mL for *M. smegmatis* and 75 µg/mL *M.tb* mutant strains. Propagation of mycobacteriophages in *M. smegmatis* mc^2155 was carried out using basal 7H10 medium supplemented with 0.4% glycerol as bottom agar and top agar containing 0.6% agar in 7H9 media supplemented with 0.2% dextrose.

Table 1: Bacterial strains used in this study

Strain	Organism	Family/ Origin	Drug resistance profile / Type
Dh5α	*Escherichia coli*	Invitrogen, USA	Chemically competent
hbhA/mtp AES phagemid	*Escherichia coli*	Jacobs Laboratory, AECOM, New York	hygromycinB resistant
mc^2155	*Mycobacterium smegmatis*	Jacobs Laboratory, AECOM, New York	Drug susceptible
V9124	*Mycobacterium tuberculosis*	F15/LAM4/KZN, Tugela Ferry, KwaZulu-Natal, South Africa	Drug susceptible

2. Construction of single and double mutant strains by specialized transduction

In constructing the V9124 Δ*hbhA-mtp* double mutant strain, a marked *hbhA* single mutant strain was generated by specialized transduction as previously described (Bardarov et al., 2002). A high-titre phage containing a targeting gene-specific AES was used to remove the majority (~80%) of the *hbhA* coding sequence (CDS) from the genome of *M. tuberculosis* V9124 (Fig. 1). Subsequently, the single Δ*hbhA* mutant strain, was confirmed by PCR and Sanger sequencing and unmarked using the unmarking phage *phAE280* (encoding a γδ-resolvase gene) to remove the hygromycin-*sacB* cassette region of the inserted AES. The unmarked Δ*hbhA* strain was confirmed by PCR and Sanger sequencing, followed by a second round of specialized transduction using an *mtp* high-titre phage containing a targeting gene-specific AES, to construct a double knockout mutant. Putative Δ*hbhA-mtp* double mutants were confirmed by PCR and Sanger sequencing, and complemented using episomal plasmids to restore single gene function in the double mutant strain to construct 2 independent complemented strains.

2.1. Preparation of high-titre phages

Plasmid DNA (kindly provided by the W.R. Jacobs Laboratory, AECOM) was electroporated into competent, actively growing *M. smegmatis* mc^2155 (Larsen et al., 2007), to select phage plaques for amplification to use in specialized transduction. Single plaques were used to prepare high-titre phage, harvested from plates with a lacey appearance (1000 plaques/plate). Additionally, a high-titre phage stock aliquot of the unmarking phage *phAE280* (encoding a γδ-resolvase gene) was also kindly provided by the Jacobs laboratory. This phage targets γδ-resolvase sites flanking the hygromycin-*sacB* cassette within the AES, in order to remove this inserted fragment to allow for an unmarked mutant to be generated, without any additional foreign DNA.

2.2. Specialized transduction

Briefly, a log-phase culture of either V9124 (to generate the Δ*hbhA* single mutant) or Δ*hbhA* (to generate the Δ*hbhA-mtp* double mutant) was washed with mycobacteriophage wash medium and resuspended in 1 mL mycobacteriophage (MP) buffer. An equal amount of pre-warmed (37 °C to 39 °C) high-titer *hbhA* phage or *mtp* phage (titre ~10^{-10} plaque forming units) was added followed by overnight incubation at 37 °C. After centrifugation, pelleted samples were re-suspended in 0.2 mL Middlebrook 7H9 broth. The total volumes were plated onto 7H10 selective plates containing 75 µg/mL hygromycin and incubated for 3 to 4 weeks at 37°C. Single colonies were screened by picking and patching simultaneously onto 7H10 agar plates with and without 75 µg/mL hygromycin.

3. Mutant confirmations:

Δ*hbhA* single mutant and Δ*hbhA-mtp* double mutant

High molecular weight genomic DNA (gDNA) of putative knockout mutants was isolated using the CTAB (NaCl-cetyl trimethylammonium bromide) method (Ausubel et al. 1989, Larsen et al., 2007) for all confirmations including PCR, Sanger sequencing and whole genome sequencing. Primers (Inqaba Biotechnology (Pretoria, South Africa) and Integrated DNA Technologies (Illinois, USA) were designed using Primer3 (Rozen and Skaletsky, 2000), with gene sequences retrieved from Tuberculist. The primers

used for confirmations of the knockouts and generation of the complementation plasmids are listed in Table 2.

Table 2: Primers used for PCR confirmation, Sanger sequencing and complementation

Gene/Amplicon Target	Forward/Left Primer	Reverse/Right Primer	Product Size (*bp*)
***mtp* whole gene**	5' CTCATGGGTCACAGCGAGTA 3'	5' ATGACAGGTTCCCTTCAAGC 3'	583
***kan* (kanamycin from pJV126)**	5' TTATGCCTCTTCCGACCATC 3'	5' GCCTGAGCGAGACGAAATAC 3'	223
***hyg* (hygromycin from GNF phages)**	5' ACCCCCCATTCCGAGGTCT 3'	5' CCGGAAGGCGTTGAGATGCA 3'	300
***sacB*-up**	5' CGGCAGGTATATGTGATGGG 3'	use with *LL*-primers or *hyg-out*	
***hyg*-out**	use with *RR*-primers or *sacB*-out	5' AACTGCTCGCCTTCACCTTC 3'	
Universal *Uptag* (GNF phages)	5' GATGTCTCACTGAGGTCTCT 3'	use with *LL*-primers	
Universal *Downtag* (GNF phages)	use with *RR*-primers	5' CGAGTGTCTGGTCTCGTAG 3'	
LL-hbhA	5' CGACATCGGCAGCTTCATCAGG 3'	use with Universal *Uptag* primer	700
RR-hbhA	use with Universal *Downtag* primer	5' TGATTTCGGCGTACTGCCAGTG 3'	770
LL-mtp	5' CCCGGCACTTGGATGCATTC 3'	use with Universal *Uptag* primer	700
RR-mtp	use with Universal *Downtag* primer	5' CGGCGTCGCCAGTGGACC 3'	850
***hbhA* whole gene (complementation)**	5’ TTTTTTGAATTCATGGCTGAAAACTCGAACAT 3’	5’ TTTTTTAAGCTTCTACTTCTGGGTGACCTTCT 3’	624
***hbhA* whole gene (sequencing)**	5' CCCAACGTCCAGACCAAAGA 3'	5' GGAGTCGATGGTGATTCGGA 3'	1313

3.2. PCR screening of putative knockout mutants

To confirm replacement of the target *hbhA* and *mtp* genes with the *hygR-sacB* cassette in both the single and double mutant strains, a 300bp hygromycin region from the inserted cassette was amplified. Putative positive mutants were also subjected to further PCR using gene-specific primers to confirm the loss of genes in the mutant strains. PCR was also carried out using the *LL-Uptag* combination of primers to amplify regions across the junction of AES insertion. A gene-specific *LL* forward primer and a universal reverse *Uptag* primer confirmed the placement and presence of the AES in the target *hbhA* and *mtp* genes when paired together (Fig.1). The relevant forward and reverse primer pairs were used for these confirmations as per Table 2.

For all PCR analyses, standard PCR amplification was carried out in 10 µL reactions using the *Taq* DNA Polymerase dNTPack PCR reagent kit (Roche Applied Sciences, Germany) under the following thermocycling conditions in the GeneAmp PCR System 9700 (Applied Biosystems): an initial enzyme activation step at 94 °C for 2 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 50-65 °C for 5 seconds (specific to the primer sets used, as per Table 2) and extension at 72 °C for 45 seconds, with a final extension at 72 °C for 10 minutes. PCR products in Novel Juice DNA loading buffer and the appropriate GeneRuler DNA Ladder (Thermo Scientific, USA) were resolved by electrophoresis on 1.2 % (w/v) agarose in 1x TBE and visualized using the ChemiDoc Gel Imaging System (Bio-Rad Laboratories, USA).

3.3. *Sanger sequencing of confirmatory PCR amplicons*

PCR was carried out as described above for the purpose of Sanger sequencing using *LL-Uptag* primer pairs (described above) for the confirmation of both Δ*hbhA* and Δ*hbhA-mtp*. Amplicons were purified using the PureLink PCR Purification kit (Invitrogen, USA). Briefly, the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, USA) was used to complete termination reactions prior to both forward and reverse sequencing on the Applied Biosystems 3130xl Genetic Analyzer platform using the *LL*- and *Uptag* primers used to generate the target amplicons. Sample preparation and sequencing was performed at the Africa Health Research Institute, AHRI (previously the Africa Centre for Population Studies, UKZN). Data was received, trimmed, a consensus sequence generated and aligned

against the H37Rv reference sequence (Cole, et al., 1998 - GenBank accession number AL123456.3) using the Geneious Pro software (V. 5.6.5).

4. Unmarking of the ΔhbhA single mutant strain - phAE280 transduction

The *phAE280* phage (titer ~10^{-10} plaque forming units) was amplified, harvested as described above and used to unmark the confirmed V9124 Δ*hbhA* mutant strain by specialized transduction. Putative transformants were plated out onto 7H10 with 10% sucrose for counter-selection. Putative positive colonies (unable to grow in the presence of 75 µg/mL hygromycin, but able to grow in the presence of 10 % sucrose) were further screened by colony PCR as described above for the absence of the hygromycin cassette. Positive unmarked mutants were also subjected to further PCR confirmation using gene-specific primers to confirm the loss of genes in the mutant strains, the *LL-uptag* combination of primers specific for the *hbhA* gene, as well as a gene-specific *hbhA*-flanking region set of primers to amplify across the whole *hbhA* gene and the flanking regions (Table 2). Sanger sequencing was also performed as described above using the gene-specific *hbhA*-flanking region primers.

5. Construction of the complementation plasmid pMV261-hbhA

The knocked-out gene (*hbhA*) was cloned into the *pMV261* episomal vector under the control of the *E.coli hsp60* promoter for complementation of the double mutant strain (Stover et al. 1991). The whole codon for the *hbhA* gene was amplified using primers incorporating *EcoRI* and *HindIII* restriction sites (Table 2) in 25 µL PCR reactions (as described above) using ~40 ng wild-type V9124 gDNA as a template and an annealing temperature specific to the primers, as per Table 2.

After electrophoresis and purification with the QIAquick Gel Extraction Kit (Qiagen, USA), the PCR product and vector were double-digested with the restriction enzymes *EcoRI* and *HindIII* (Thermo Scientific, USA) for 1hr at 37 °C. The linearized vector was treated with shrimp alkaline phosphatase (SAP). The digested PCR product and SAP-treated vector were purified using the PureLink PCR Purification kit (Invitrogen, USA) before the gene was ligated into the multiple cloning site (MCS) of the linearized vector with T4 DNA ligase (Thermo Scientific, USA). The ligation reaction was then transformed by heat shock into competent Dh5α *E. coli* cells (Invitrogen, USA) and

plated for selection onto LB agar plates containing kanamycin (50 µg/mL). Plasmid DNA of single colonies grown in LB broth with kanamycin was isolated using the Qiagen Mini-Prep Plasmid Purification Kit (Qiagen, USA).

Clones were screened by PCR amplifying and sequencing the *hbhA* target gene, using plasmid DNA and the primers used to generate the whole *hbhA* gene plasmid insert (Table 2). Putative positive plasmids were also subjected to restriction digestion of the purified plasmid with the restriction enzymes *EcoRI* and *HindIII* (Thermo Scientific, USA) to confirm the insert. Plasmids were additionally Sanger sequenced by Inqaba Biotechnology using primers designed by them specific to the *pMV261* plasmid backbone.

6. Harvesting of the pMV261-mtp complementation plasmid from the complemented mtp-mutant strain

In order to obtain stock of the *mtp* complementation plasmid, total gDNA was isolated as above from the *M.tb* V9124 complemented *mtp*-mutant (Ramsugit et al. 2013), and subject to plasmid purification using the Qiagen MiniPrep Plasmid Purification kit (Qiagen, USA). Purified samples were quantified and confirmed by gel electrophoresis as described above. Samples were transformed into competent Dh5α *E.coli* cells (Invitrogen, USA) and plated on LB agar plates containing the selective antibiotic kanamycin at a concentration of 50 µg/mL. Individual single colonies were grown in LB broth with kanamycin, and plasmid DNA was isolated using the Qiagen Mini-Prep Plasmid Purification Kit (Qiagen, USA). Clones were screened by PCR using plasmid DNA, kanamycin-targeting primers and the primers used to generate the *mtp* insert (Table 2).

7. Complementation of ***Δ****hbhA and* ***Δ****hbhA-mtp mutants with their respective genes to confirm restoration of gene function*

Mutant strains were electroporated with plasmid constructs as per Larsen et al. (2007). Electroporated cells were selected for on 20 µg/mL kanamycin-containing 7H11 agar plates, following which single colony transformants were inoculated into 7H9 liquid media, and screened by colony PCR (as described above) for the presence of the kanamycin resistance cassette and the target genes (either *hbhA* or *mtp*).

8. In vitro growth kinetics (OD_{600nm}, CFU count, REMA viability assay)

Aliquots (500 µL) of a stock culture stored at an OD_{600nm} of 1, equivalent to ~1 X 10^8 cells/mL (Larsen et al., 2007), were inoculated into 50 mL of 7H9 broth (supplemented with 10 % OADC, 0.05 % Tween-80 and 0.5 % glycerol). After growth with shaking at 37 ºC for 6 to 8 days, cultures at an OD_{600nm} of 1.6-1.8 were centrifuged at 3000 RPM for 10 minutes. The pellet was washed twice with 8 mL of PBS-T at 4000RPM for 10 minutes and re-suspended in 1 mL of supplemented 7H9 broth. The culture was back diluted to an OD_{600nm} of 0.015 in 25 mL of supplemented 7H9 broth in triplicate and grown with shaking at 37ºC for 30 days for measurements for OD_{600nm}, CFU counts and metabolic activity (resazurin microtitre assay) at baseline & every 3rd day over a 21-day incubation at 37 ºC. All assays were performed in triplicate for technical and biological repeats.

OD readings were measured in triplicate using a spectrophotometer (Biochrom WPA Lightwave II, Labotec) at a wavelength of 600 nm. CFU counts were performed by plating out 100µL of a 10-fold serial dilution of culture in triplicate on 7H11 plates containing 10 % OADC and 0.5 % glycerol. Colonies were counted after incubation at 37 ºC for approximately 21-25 days.

Viability was evaluated by quantifying the metabolic activity of 100µL of broth culture using the resazurin microtiter assay (REMA) in a 96-well microtitre plate in triplicate. The blank negative control was 100 µL broth. After the addition of 15 µL of a 0.02 % resazurin solution (Sigma-Aldrich, USA), the plate was incubated overnight at 37 °C and OD readings measured at 600 nm in a GloMax®-Multi Detection System absorbance spectrophotometer (Promega Corp., USA).

9. Crystal violet quantification of biofilm formation

Strains were grown to OD_{600nm} 1 and diluted 1:100 (v/v) in Sauton's minimal medium. A suspension of 2 mL was cultured for biofilm growth in 24-well plates incubated at 37 °C in 5 % CO_2 for 5 weeks without shaking. Following incubation, the spent media was carefully aspirated to prevent disturbance of the biofilm layer, and the dried cell biomass was allowed to air dry in the biosafety cabinet. Individual wells were incubated with 0.5 mL of 1 % crystal violet solution for 10 minutes. Wells were washed three

times with deionized water and the biomass was allowed to dry, following which 2.5 mL of a 95 % ethanol solution was added to each well. Wells were incubated for 10 minutes and the absorbance of the aspirate was measured at A_{600} in a GloMax®-Multi Detection System absorbance spectrophotometer (Promega Corp., USA). All assays were performed in triplicate for both technical and biological repeats.

10. Statistical analysis

All experiments were performed in independent triplicate biological experiments with triplicate technical repeats. Data were analyzed by one-way Analysis of Variance (ANOVA), with the statistical analysis performed using SPSS 25.0 statistical software, and a *p*-value less than 0.05 considered significant. Error bars represent the standard error of the mean (SEM) of these triplicate biological and technical repeats.

Results:

1. Confirmation of the *hbhA* single mutant, Δ*hbhA*

Positive *hbhA* single mutant strains were purified and unmarked as described above to generate gene organization as per Fig. 1 A. High molecular weight gDNA was extracted and used to carry out the PCR for the *hbhA LL-uptag* fragment and the *hyg* target in the AES to confirm the partial removal of the *hbhA* gene (Fig. 2 A and B respectively). Sanger sequencing was performed on putative unmarked *hbhA* mutant strains using flanking region primers to generate a 1313bp *hbhA* whole gene amplicon (Fig. 3 A).

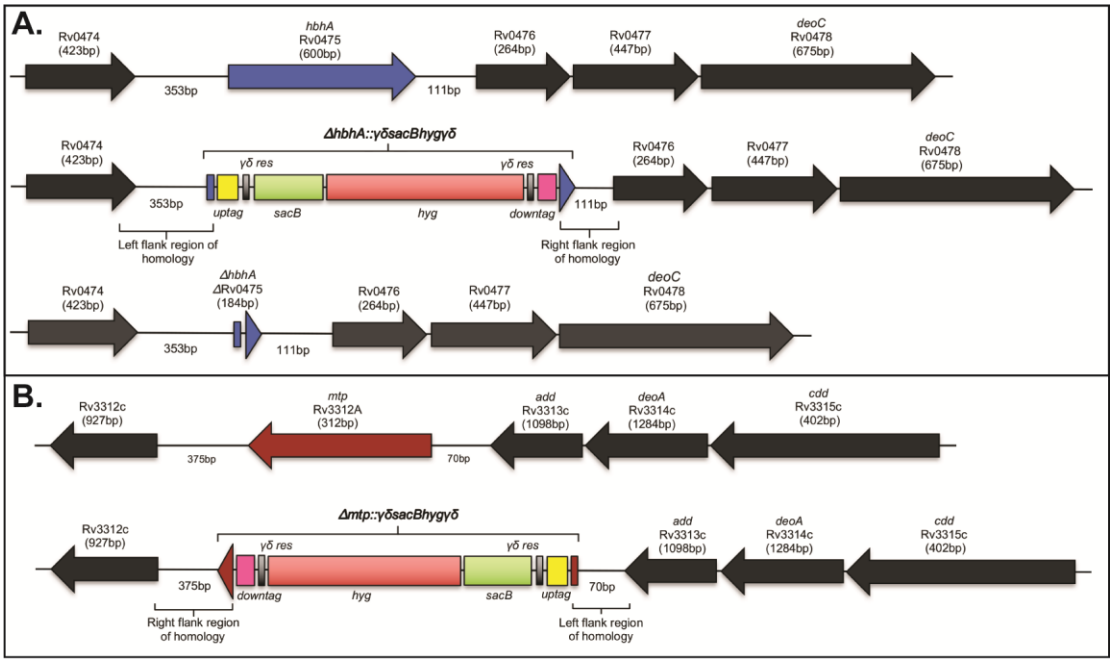

Fig. 1: Gene organization maps of double gene knockout mutants indicating the resulting allelic exchange replacement in the ORFs. A. The unmarked *hbhA* forward direction gene arrangement and B. The marked *mtp* reverse direction gene arrangement, specific to the double *hbhA-mtp* mutant. Maps indicate respective flanking regions, regions of gene deletion and sites important to primer design for confirmation and unmarking techniques.

2. Construction and confirmation of the *hbhA-mtp* mutant strain, ΔDM

Specialized transduction of the unmarked V9124 *hbhA* single mutant strain with a *phAE159* shuttle phasmid carrying an *mtp* AES was performed as described by Bardarov et al. (2002) to generate gene organization depicted in Fig. 1 B. Transduced cells were plated onto hygromycin selective media and colonies were screened by PCR for the presence of multiple regions to confirm the insertion of the AES cassette.

Amplification of the *hbhA LL-uptag* fragment and the *mtp LL-uptag* confirmed the partial removal of the *hbhA* and *mtp* genes (Fig. 2 C and D respectively).

The *LL-uptag* primer sets amplified a region spanning the left flanking arm and a region upstream of the AES *hyg-sacB* cassette. The *hbhA LL-uptag* and the *mtp LL-uptag* primer sets were used to amplify regions showing the presence of the AES that was inserted into the genome at the specific regions (in the *hbhA* CDS and *mtp* CDS respectively), thus indicating the generation of a *hbhA-mtp* double mutant strain. These PCR's both served to confirm the *hbhA-mtp* double mutant.

Amplicons for PCR positive strains were purified and subjected to Sanger sequencing which further confirmed the double mutant strain (Fig. 3 B and C). Additionally, gDNA was subject to whole genome sequencing by our collaborators at the William R. Jacobs laboratory at AECOM on the Illumina MiSeq platform confirming Sanger sequencing results (data not shown).

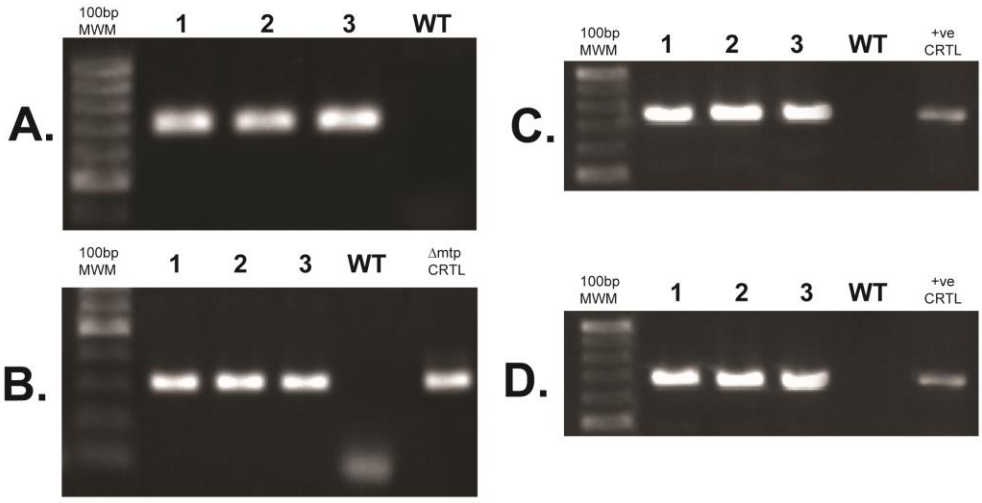

Fig. 2: PCR confirmation of *hbhA* single and *hbhA-mtp* double knock out mutant strains. A. 770bp *hbhA* LL-uptag amplicon indicates the Δ*hbhA* single mutant in 3 putative colonies B. 300bp *hyg* amplicon confirms the 3 putative colonies as Δ*hbhA* single mutant strains. C. 770bp *hbhA* LL-uptag amplicon indicates the presence of the *hbhA* AES in 3 putative Δ*hbhA-mtp* double mutant strains; D. 700bp *mtp* LL-uptag amplicon indicates the presence of the *mtp* AES in 3 putative Δ*hbhA-mtp* double mutant strains. Frames C & D together confirm the generation of the Δ*hbhA-mtp* double mutant, ΔDM.

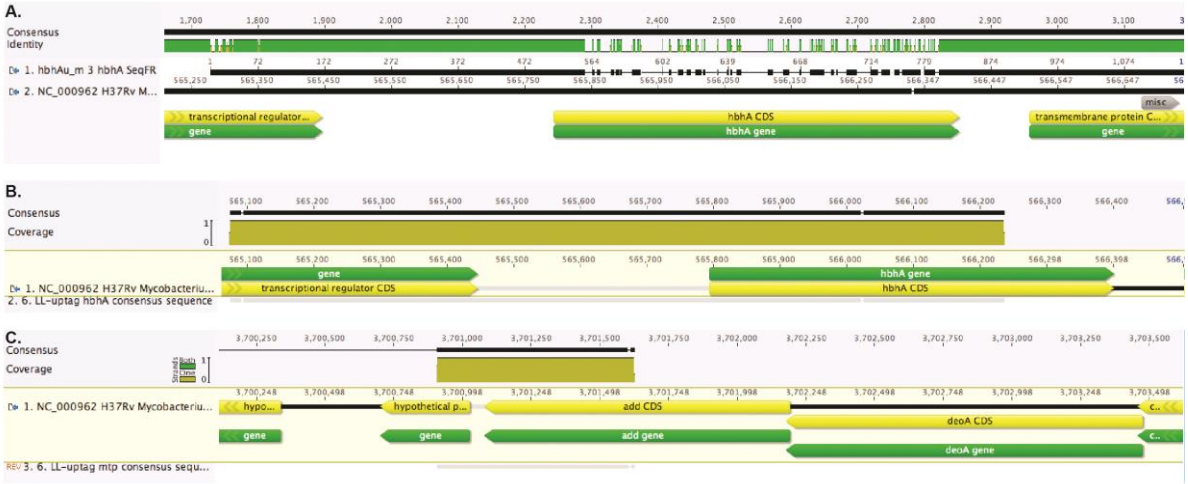

Fig. 3.: Sanger sequencing alignment confirmation. The H37Rv whole genome was used as a reference strain to align purified sequenced PCR amplicons against. Alignment of the amplicons agains the correct regions and loci serves as confirmation of unmarking or deletion of genes. A.1313bp *hbhA* whole gene amplicon sequencing confirms *phAE280* unmarked *hbhA* mutant. B. 770bp *hbhA* LL-uptag amplicon confirms presence of the remaining section of the *hbhA* AES (post-unmarking) in the double mutant. C. 700bp *mtp* LL-uptag amplicon confirms *mtp* absence in the double mutant.

3. Complementation of double mutant

3.1.Construction of the complementation plasmid pMV261-hbhA

To complement and restore the expression of the *hbha* gene in the mutant strains, the whole gene CDS of *hbhA* was ligated into the MCS of the episomal expression plasmid *pMV261*, and transformed into competent *E.coli* Dh5α cells. Plasmids isolated from the resultant transformants on kanamycin selective media, were screened using restriction digest (*EcoRI* and *HindIII*), PCR and Sanger sequencing. Figure 4A confirms the insertion of the *hbhA* whole gene into the *pMV261* plasmid backbone by restriction digest. Confirmed plasmids were then subject to PCR using the primers initially used to amplify the whole *hbhA* gene. Forward and reverse Sanger sequencing of these positive amplicons was performed to confirm the whole gene insert were present in the plasmid *pMV261-hbhA* (Fig. 4B).

Fig. 4: Complementation of the *hbhA-mtp* ΔDM with *pMV261-hbhA* and *pMV261-mtp* A. Three positive clones of *pMV261-hbhA* confirmed by *EcoRI-HindIII* restriction digest. B. Positive clone of *pMV261-hbhA* confirmed by Sanger sequencing alignment against the H37Rv whole genome as a reference strain. Alignment of the amplicons against the correct regions and loci serves as confirmation of the correct gene being inserted into the plasmid backbone for gene expression. C. Presence of the 223bp kanamycin cassette amplicon confirm successful electroporation of the Δ*hbhA-mtp* double mutant, ΔDM, by *pMV261-hbhA* (lanes 1-3) and *pMV261-mtp* (lanes 4-6).

3.2. Generation of complemented double mutant strains

Mutant strains were electroporated with the *pMV261-hbhA* (described above) and *pMV261-mtp* plasmid (Ramsugit et al., 2013) constructs as per Larsen et al. (2007). PCR was performed on single colony transformants to screen for the presence of the kanamycin resistance cassette (Fig. 4C), confirming successful complementation of the *hbhA-mtp* double mutant strain with either *pMV261-hbhA* or *pMV261-mtp*.

4. HBHA and MTP significantly impact in vitro growth kinetics but not viability

4.1. HBHA and MTP contribute jointly to in vitro growth of wildtype V9124 strain

OD_{600nm} measurements demonstrated significant differences among the strains across the 21-day period ($p < 0.01$) with the WT strain showing consistently the highest growth at all intervals (Fig. 5 A). Although the collective differences in growth was statistically significant [F (5,12) = 46.601, $P < 0.01$], the major significance was seen when comparing V9124 to any of the strains. The ΔDM showed significant growth differences relative to the complemented strains from day 12 onward ($p < 0.05$).

No significant differences were seen in the CFU/mL among all the strains on day 0, in contrast to the significant differences noted between ΔDM and WT on day 3 ($p = 0.006$) and day 6 ($p=0.003$) (Fig. 5 B). Significantly higher growth of the WT ($p = 0.00$) relative to all the strains was observed from day 9 to 21, and between ΔDM and the complemented strains on day 21 ($p < 0.01$).

4.2. HBHA and MTP do not affect viability of V9124 strain

REMA showed overall viability did not differ significantly among strains (Fig. 5 C). On day 6 however, the viability of the ΔDM *mtp*-complement was significantly higher (mean 1.560 ± 0.05604; $p=0.041$) in comparison to the ΔDM. The viability of the ΔDM and complemented strains on day 21 was significantly increased relative to the WT V9124 ($p < 0.05$).

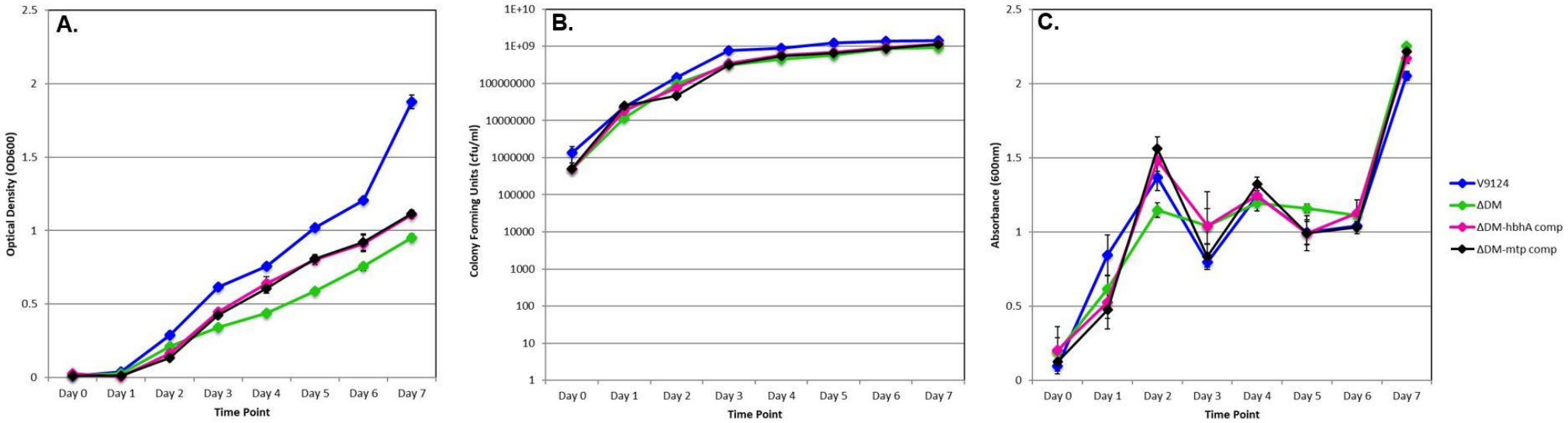

Fig. 5.: Growth kinetics & viability assay: optical density measured at 600 nm, colony forming units by serial dilution plate out, and metabolic viability determination by rezasurin (Alamar Blue dye) reduction. Error bars are indicative of the standard error of the mean (SEM) for triplicate technical and biological repeats. V9124 wildtype strain was grown alongside the *hbhA-mtp* double mutant (ΔDM) and the complemented strains (ΔDM-*hbhA* comp; ΔDM-*mtp* comp) over 21 days. Growth measurements were captured every 3rd day and data were analyzed by one-way analysis of variance (ANOVA) using SPSS 25.0 statistical software. A. Optical density measurements at OD_{600nm} indicate a significant ($p<0.05$) difference in the growth of the double mutant relative to the growth of the wildtype strain. Growth was recovered by ~50 % when the double mutant was complemented to restore individual genes. B. CFU counts indicate a significant ($p<0.05$) difference in the growth of the double mutant relative to the growth of the wildtype strain after timepoint 2. Growth was recovered slightly when the double mutant was complemented to restore individual gene function. C. The REMA assay was performed to establish viability, and indicated no significant ($p>0.05$) differences in the viability of the double mutant relative to the wildtype strain over the 21-day period.

3.4. Biofilm assay: Biomass quantification by crystal violet staining

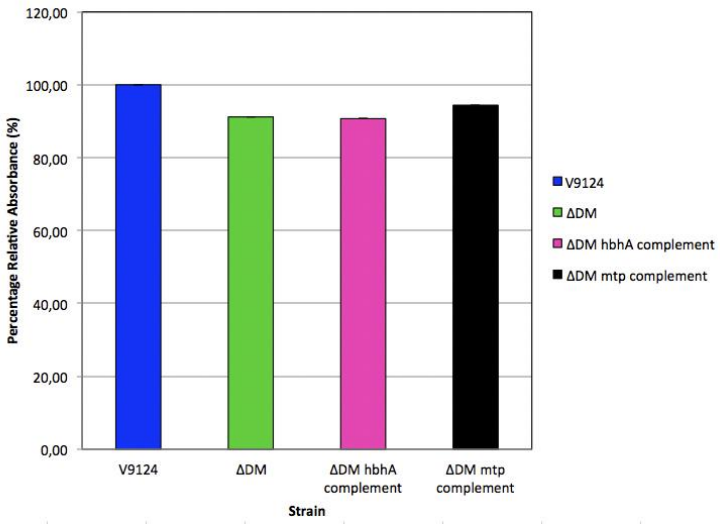

Fig. 6: Biofilm production assessed in mutant and complemented strains by relative quantification of biomass production. The V9124 wildtype strain was grown alongside the *hbhA*-*mtp* double mutant (ΔDM) and the complemented strains (ΔDM-*hbhA* comp; ΔDM-*mtp* comp). The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 25.0 statistical software; $F(5,12) = 3.582$, $P < 0.05$. Error bars are indicative of the standard error of the mean (SEM) for triplicate technical and biological repeats.

Crystal violet quantification of biofilm production demonstrated an 8.82% reduction by the ΔDM and 9.3% reduction by the ΔDM *hbhA* complemented strains relative to V9124 (Fig. 6). Even though these differences did not vary enough in the mutants relative to the wildtype strain to warrant any significance, MTP does appear to play more of a role than HBHA in biofilm formation, with the *mtp* complemented double mutant showing only a 5.6% reduction relative to the V9124 wildtype strain.

Discussion:

The presence of adhesins on the bacterial surface is crucial to the survival of pathogenic strains, as these molecules are fundamental in instigating and perpetuating host-pathogen interaction (Solanki et al., 2018). The ability to specifically target the initial interaction between host and pathogen in order to prevent infection holds great promise for the identification of novel biomarkers for rapid TB diagnostic tools, drugs and vaccine development. Therefore, there is a need to fully characterise the function of the proteins/putative proteins that have been identified to play a role in this primary contact, as this area of study is still relatively in its infancy.

Amongst the several adhesins (Díaz-Silvestre et al., 2002; Kinhikar et al., 2006; Alteri et al., 2007; Ragas et al., 2007; Hickey et al., 2010; Kumar et al., 2013; Esparza et al., 2015) identified in *M.tb*, two molecules, HBHA (Pethe et al., 2001) and MTP (Alteri et al. 2007, Ramsugit et al., 2013) have emerged as significant potential biomarkers that may be targeted for intervention strategies. Therefore, using functional genomics, this study attempted to quantify the combined contribution of these genes in growth kinetics, viability and biofilm formation, by comparing with a double *hbhA-mtp* knockout mutant (ΔDM) and complemented strains against the wildtype V9124 *M.tb*.

The functional copies of the genes encoding the two major adhesin molecules, Rv0475 (*hbhA*) and Rv3312A (*mtp*), were knocked-out of V9124 successively using the phage-mediated method of mycobacterial specialized transduction (Larsen et al., 2007). Approximately 80% of the coding sequences of these two genes were removed from the genome, rendering the encoded proteins non-functional in the ΔDM strain. To quantify the relative contribution of each adhesin, *hbhA* and *mtp* complemented strains were separately constructed in ΔDM. A complementation plasmid was created to express *hbhA* episomally, whereby the whole *hbhA* gene was ligated downstream of the *hsp60* promoter in the episomal Kan+ plasmid, *pMV261*, and electroporated into ΔDM. Thereafter, the *mtp* complementation plasmid *pMV261-mtp* (extracted from the complemented Δ*mtp* mutant strain previously generated by Ramsugit et al., 2013), was electroporated into ΔDM to generate an *mtp*-complemented strain.

The outcomes of the growth assays, by means of OD_{600nm} quantification and CFU counts suggests that HBHA and MTP in concert, significantly contribute to growth of *M.tb*. Moreover, growth of the independently complemented double mutant strains suggests that this contribution is approximately equal for each adhesin.

The *in vitro* data also revealed that whilst the growth rate was significantly impaired, the double mutant strain remained metabolically viable compared to the wild type and complemented strains, suggesting that HBHA and MTP do not play a role in the metabolic activity of *M.tb*. However, the REMA assay may not be sensitive enough to show this conclusively, and a more definitive test is required. It is possible that variability in the data could be due to this lack of sensitivity, when measurements are performed much later during the growth cycle. This may be due to the larger numbers

of bacterial cells present in the wild type compared to the mutant and complemented strains.

Formation of biofilms, which are known to increase antibiotic resistance and reduce clearance from the host, is regarded as being highly dependent upon expression of bacterial adhesion molecules (Stones and Krachler, 2015). Although no statistically significant differences were seen between the different strains in terms of biomass formation, the removal of *hbhA* and *mtp* concurrently caused an 8.82% reduction in biomass relative to the wild type V9124. The notable reduction in biomass of the ΔDM *hbhA* complemented strain that still harboured the *mtp* gene, and the lower, insignificant reduction by the ΔDM *mtp* complemented that retained *hbhA* relative to V9124 support the findings by Ramsugit et al. (2013), and suggest that MTP may potentially have a greater effect on biofilm formation that HBHA. Variability in the degree of biomass reduction presented in the two studies is notably high, however, this may be attributed to the effect of restoring a major adhesin with an episomal plasmid. This may not be enough to appropriately regulate the expression pathways responsible for control of compensatory mechanisms that may have been switched on in the double mutant, with a loss of gene function. Upregulation of other genes may also interfere with the feedback mechanisms to produce unexpected phenotypes and a ripple effect on regulation pathways. Pathway analysis and additional studies using the double and single mutants to establish biomass and viability are thus valuable in painting a true picture of why there may be discrepancies between the two studies, and if this is indeed the case.

The study outcomes indicate the key pathogenic importance of the two major *M.tb* adhesins in promoting *in vitro* growth of the F15/LAM4/KZN susceptible strain. These results suggest the significance of the two adhesins in combination as biomarkers for the development of potential diagnostic interventions. Treatment strategies targeting multiple adhesins may prove most beneficial to incapacitating the pathogen, as the simultaneous absence of these adhesins may prevent the pathogen from entering the cell, thereby restricting their ability to multiply successfully, resulting in a potential for reduced growth capacity, *in vivo*. Adjunctive therapy with blocking antibodies to prevent bacilli entry into the cell may also serve to boost efficacy of the current first line drugs.

Although the general conceptual appeal of a targeted blocking antibody in vaccine development remains popular, anti-adhesion therapies are still not commonly utilized in the treatment of bacterial infections. This is due to the expression of multiple adhesion molecules in a well-modulated time- and tissue-specific manner over the course of an infection (Stones and Krachler, 2015). Additionally, bacteria are able to initiate compensatory mechanisms to circumvent the effects of a loss of expression of key genes, and possess a host of molecules that either have moonlighting function as adhesins outside of their core functions (Govender et al., 2014), or work via tributary pathways to facilitate entry into the host (e.g. host scavenger receptors, host complement receptors and host Fe receptors) (Zimmerli et al., 1996). However, a large portion of the *M.tb* genome encodes hypothetical proteins that have yet to be studied and functionally characterized (Mazandu & Mulder, 2012), making it most likely that the organism possessed genes encoding novel factors that hold roles in host cell adherence that are still unidentified (Mann et al., 2016). The presence of the large number of predicted adhesins (as reviewed in Govender et al., 2014) raises the further issue of redundancy and functional duplication, a potential confounding factor in gene knockout studies. This study highlights the usefulness of targeting multiple adhesins as a way to effectively counteract this pitfall.

In generating the double mutant, the study was able to illustrate a potential compounded effect caused by a loss of function of two major adhesins on bacterial growth. A limitation of this study design was the independent complementation of the double mutant strain by the single genes in episomal expression plasmids. This resulted in incomplete complementation of both *hbhA* and *mtp* together in ΔDM to fully restore gene function. The motivation for generating these individual single complemented ΔDM strains was to attempt to evaluate the individual contribution of each adhesion molecule in the assays done, to establish if one gene had variable or increased effects on growth, viability and biofilm formation over the other.

Even though the outcomes of these experiments allude to the contribution toward growth phenotypes being approximately equal for each adhesin, it must be considered that a number of factors could have impacted the effective complementation of the double mutant, such as transcriptional errors in the development of the expression plasmid or reduced activity of the plasmid promoter. However, in order to fully

understand the combined effect of these genes together, further studies utilizing a double-complemented *hbhA-mtp* mutant strain are needed as a supplementary step to substantiate and truly validate the outcomes of this study, to see the full restoration of gene function when both genes are complemented.

Further studies looking into the impact of the absence of these genes on signaling pathways and gene regulation networks (both in *M.tb* and the host) would shed more light into the role they play in facilitating bacterial proliferation, and the potential effect that they may have on host immune modulation. They may also serve to indicate potential compensatory genes and mechanisms (for example, those highlighted in Zimmerli et al., 1996) to be considered as novel therapeutic targets for development alongside *hbhA* and *mtp*.

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Conflict of interest:

None to declare.

Ethical statement:

Ethical clearance was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (REF: 257/13).

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The second paper evaluated the potential importance of HBHA and MTP as a combined target for therapeutic drug and vaccine development by establishing the *in vitro* growth, viability and biofilm formation capacity.

A novel double mutant strain deficient in both the *hbhA* and *mtp* genes was generated in the susceptible F15/LAM4/KZN *M. tuberculosis* strain V9124, and single gene complemented strains were then derived. Growth assay data showed the concurrent removal of both major adhesin genes results in a dramatically significant reduction in growth, while cellular viability and biofilm formation remained unaffected. This confirmed the potential novel efficacy of targeting *hbhA* and *mtp* and their protein counterparts HBHA and MTP for drug and vaccine development.

Following from this, the next paper aimed to further establish the contribution of these two protein molecules to the *in vitro* adhesion to and invasion of A549 pulmonary epithelial cells.

CHAPTER 4: Paper 3 – "Mycobacterium tuberculosis heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) proteins facilitate adhesion to and invasion of A549 pulmonary epithelial cells."

Title: *Mycobacterium tuberculosis* heparin binding haemagglutinin adhesin (HBHA) and curli pili (MTP) proteins facilitate adhesion to and invasion of A549 pulmonary epithelial cells.

Running title: *M.tb* HBHA and MTP facilitate adhesion and invasion of epithelial cells

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Abbreviations: TB – tuberculosis; *M.tb* – *Mycobacterium tuberculosis*; HIV/AIDS – Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome; HBHA/*hbhA* - heparin binding haemagglutinin adhesin; MTP/*mtp* - *M.tb* pili protein; ΔDM - *Δhbha-mtp* double mutant; BCG - Bacillus Calmette-Guérin ; WHO - World Health Organization; POC – point of care; kDa – kilodalton; THP-1 - Tohoku Hospital Pediatrics-1 (human monocytic acute leukemia cell line); A549 - human non-small cell adenocarcinoma pulmonary alveolar epithelial cells; AES - allelic exchange substrates; RPM – revolutions per minute; FBS – foetal bovine serum; PBS – phosphate buffered saline; OADC - oleic acid-albumin-dextrose-catalase enrichment; CFU – colony forming units; DMHC – double mutant *hbhA* complement; DMMC – double mutant *mtp* complement; SEM- standard error mean; ANOVA - one-way analysis of variance; KZN – KwaZulu-Natal; GAPDH – glyceraldehyde-3-phosphate dehydrogenase

Abstract:

Novel targets are urgently sought for the development of therapeutic interventions to reduce high global TB burdens. Several studies have demonstrated the importance of the two primary *M. tuberculosis* (*M.tb*) adhesins, the heparin binding haemagglutinin adhesin (HBHA) and the *M.tb* curli pili protein (MTP) independently in pathogenesis, suggesting that they may be suitable biomarkers for this purpose. This study aimed to elucidate the role of the two adhesins simultaneously in the host –pathogen interaction. Adhesion and invasion assays were performed in A549 pulmonary epithelial cells infected with a previously constructed, double knockout mutant Δ*hbha-mtp* (ΔDM) *M.tb* strain, single gene (ΔDM-*hbhA* and ΔDM-*mtp*) complemented and the wild-type strain V9124. A significant reduction in adherence to A549 pulmonary epithelial cells (81%) was seen in the ΔDM strain relative to V9124 ($p = 0.00$), while the invasive ability of this mutant strain was reduced by 83% ($p = 0.00$). Complementation with *hbhA* partially restored adhesion to 45% and invasion to 29% relative to V9124, while complementation with mtp partially restored adhesion to 43% and invasion to 40% relative to V9124. Both adhesins thus play a significant role in adhesion and invasion, with MTP having a potentially greater invasin role than HBHA in the F15/LAM4/KZN susceptible strain V9124. HBHA and MTP appear to be similar in their role as adhesins. Their major roles in the overall adhesion-invasion capacity of *M.tb* thus suggests that these two molecules, in combination, present an attractive option as novel targets for potential vaccine/drug development.

Introduction:

Tuberculosis (TB) remains a high burden disease globally, with a low likelihood to reach the specific targets set out in the End TB Strategy (including a 90% reduction in TB deaths and an 80% reduction in new TB cases per year, by 2030) (WHO, 2017). The ability of *Mycobacterium tuberculosis* (*M.tb*) to adhere to and infect host cells is core to the success of this pathogen in evading immune responses, resulting in one of the most debilitating diseases known to man. Whilst TB continues to result in approximately 1.67 million worldwide (WHO, 2017), our knowledge regarding the biology and genetics of the causative organism remains inadequate to effectively reverse this trend. The rapid escalation in the number of *M.tb* infections (an estimated 10.4 million new TB cases globally during 2016, as per the WHO Global Report 2017), facilitated by a lack of rapid point of care (POC) diagnostic tests, high prevalence of HIV-TB co-infections, together with the emergence of multi-drug-resistant strains, has fast-tracked the search for novel biomarkers and drug targets in order to reduce the high TB burdens.

The currently implemented Bacillus Calmette-Guérin (BCG) vaccine was developed almost 100 years ago and has been shown to prevent severe forms of TB in children. However, no vaccine currently exists with efficacy in preventing TB disease in adults, either pre- or post- exposure to TB infection (WHO, 2017). There is an urgent need for development of an effective vaccine, as well as innovative, efficacious drugs with sterilizing ability, to shorten the current duration of treatment and reduce the risk of patient non-compliance and relapse (Betts, et al., 2002; Krieger et al., 2012; Gaur & Mishra, 2017). Functional genomics studies are crucial in the validation of novel pathogen-specific drug targets for the development of effective new therapies (Bellinzoni & Riccardi, 2003). As with many pathogenic micro-organisms, adhesion of the infecting agent to the host surface is thought to be a crucial prerequisite in the pathogenic process of TB infection, allowing for host colonization (Espitia et al., 2012). This process is mediated by adhesin molecules (amongst others) present on the microbial surface, which serve to recognize and bind receptors on specific host cells (Nizet et al., 2017).

The host pulmonary epithelial cells are considered as the initial barrier faced by *M.tb.* upon entry into the human host, due largely to the abundance of epithelial cells in the

lung (Crandall and Kim 1991; Pessolani et al., 2003; Adlakha et al. 2012; Sarkar et al., 2016). Although the interaction between *M.tb.* bacilli and the lung epithelial cells has largely been overlooked until recently (Harriff et al., 2014), invading bacilli potentially have a greater chance of infecting these cells rather than macrophages, given their relative abundance within the alveolar space (Crandall and Kim 1991; Pessolani et al., 2003). Additionally, bacilli have a greater chance of survival within epithelial cells than in the harsh environment presented by the presence of unarrested phagosomes within the macrophage cytoplasm. These cells provide a potentially optimal niche within which the host-adapted pathogen can readily replicate if they are able to successfully evade and circumvent the host defences and immune responses (Falkow, S., 2006; Cambier et al., 2014). This is possible since they are afforded direct access to the host lymphatic and blood systems (without the need for carrier macrophages) upon disruption and destruction of the alveolar vascular endothelium (McDonough and Kress, 1995; Dobos et al., 2000; Castro-Garza et al., 2002).

Mycobacteria display multiple proteins with primary and secondary adhesin function that interact with host cell receptors (Menozzi et al., 2006). The primary adhesin in *M.tb* is a 28-kDa heparin-binding haemagglutinin adhesin (HBHA), a virulence factor that facilitates the dissemination of *M.tb* from the site of primary infection by initiating interaction with epithelial cells (Menozzi et al., 2006; Esposito et al., 2011). Extrapulmonary dissemination of bacilli is essential to the development of active TB infection (Yang & Kong, 2015; Polena et al., 2016). The protein is localized to and expressed on the surface of the bacteria, and is primarily responsible for adhesion to epithelial cells (Esposito et al., 2011). Mutant strains deficient in functional copies of *hbhA* show a reduced ability to bind epithelial cells and disseminate from the lungs to other tissues (Menozzi et al., 2006; Esposito et al., 2011).

The two physically distinct *M.tb* pili adhesin morphotypes (curli-like pili (MTP) and type IV pili) are produced during pathogenesis and play a key role in adherence to epithelial cells, in turn stimulating the humoral immune response (Alteri et al., 2007). Ramsugit et al. (2013) highlighted this role as an adherence factor, confirming the involvement of pili proteins in cellular aggregation and biofilm formation. Ramsugit & Pillay (2014) additionally established the role of MTP in the adhesion to and invasion of THP-1 macrophages (Ramsugit and Pillay., 2014) and A549 pulmonary epithelial cells (Ramsugit et al., 2016), as well as in modulating immune response in epithelial

cells (Ramsugit et al., 2016). MTP is considered crucial in mediating close interaction and colonization with host lung epithelial cells (Alteri et al., 2007). The current study undertook to assess the combined *in vitro* effect of MTP and HBHA in the adhesion to and invasion of A549 pulmonary epithelial cells, by infection with a double knockout mutant Δ*hbha-mtp M.tb* strain.

Methods:

Bacterial strains

The combined potential of HBHA and MTP proteins as epithelial cell-associated adhesins and invasins of *M. tuberculosis* was investigated using a novel double gene knock-out mutant strain, Δ*hbha-mtp* (ΔDM), generated by phage-mediated specialized transduction of allelic exchange substrates (Govender et al., unpublished).

Briefly, the *mtp* gene was disrupted by mycobacterial specialized transduction using phage-packaged allelic exchange substrates (AES) in a confirmed unmarked *hbhA* mutant strain, Δhbha::γδsacBhygγδ (Δ*hbha*) in *M.tb* V9124, a drug susceptible clinical strain of the F15/LAM4/KZN family (Govender et al., unpublished). Additionally, single gene complemented strains, ΔDM-*hbhA* complement and ΔDM-*mtp* complement were also generated by electroporation of episomal plasmids expressing the individual genes into the double mutant strain (Govender et al., unpublished). Strains were cultured in Middlebrook 7H9 medium (Difco, Detroit, MI, USA) supplemented with 10 % (v/v) OADC (oleic acid-albumin-dextrose-catalase) enrichment (Becton Dickinson, Sparks, MD, USA), 0.5 % (v/v) glycerol (Sigma, St. Louis, MO, USA), and 0.05% (v/v) Tween 80 (Sigma) and incubated with shaking (130 RPM) at 37 °C for 10-12 days to an OD_{600nm} ~0.8-1.0.

Adhesion and Invasion Assays

The methods were performed as described by Ramsugit et al. (2016). A549 adherent hypotriploid human pulmonary epithelial cells (ATCC® CCL-185™) were cultured in 75 mL vented tissue culture flasks in 20mL MEM Eagle (EMEM) with Earle's BSS, with L-glutamine (Lonza, Rockland, ME, USA) containing 10 % v/v heat-inactivated foetal bovine serum (FBS) (Gibco, USA), under 5 % CO_2 at 37 °C to confluency. Twenty-four well plates (NEST Biotechnology, China) were seeded at a density of 5 ×

10^5 cells to ensure a multiplicity of infection of 5:1 after trypsinized confluent cells were pooled and washed. Prior to infection, confluent cells in seeded plates were repeatedly washed with 1 mL 1x phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) to remove any non-adherent A549 cells and residual media, followed by the addition of 1mL fresh tissue culture medium.

Log phase bacterial cell cultures were pelleted at 2,000 × g for 20 min at 20 °C (Heraeus Multifuge 3S-R Centrifuge; Thermo Scientific, Germany) and resuspended in 10mL fresh tissue culture media. Pulmonary epithelial cells were infected in triplicate with wildtype, mutant and complemented bacterial strains at a multiplicity of infection of 5:1. Plates were incubated at 37 °C with a constant 5 % feed of CO_2 for either 1 hour (adhesion assay) and 2 hours (invasion assay) post-infection. To confirm accurate bacterial counts were used for infection, serial dilutions of the bacterial suspensions were plated in triplicate onto 7H11 agar plates to establish the CFU. Uninfected epithelial cell controls were included in all assays.

Following the respective incubation periods for the adhesion and invasion assays, the spent infection medium was aspirated and wells were washed three times with 1 mL sterile PBS. Amikacin (300 mg/mL; Sigma) was then used to treat the invasion assay plate, followed by incubation for 1 hour at 37 °C in 5 % CO_2. Subsequent killing of any adherent extracellular bacteria in the invasion assay was confirmed by plating serial dilutions of the amikacin-containing media onto 7H11 plates and enumerating CFUs after 3 weeks. Following incubations for both assays, cells were washed in triplicate with 1 mL 1x PBS, and 1 mL 0.1 % Triton X-100 (Sigma) was added to each well. Plates were incubated for 20 minutes to allow lysis of adherent A549 cells. The cell lysates were serially diluted in PBS, plated in triplicate onto 7H11 agar plates, and incubated at 37 °C in 5 % CO_2 for 3 weeks to determine the number of adherent or invading bacteria.

The degree of adhesion or invasion was determined as a percentage and calculated as: (colony-forming units (CFU) of the attached or invaded bacteria/CFU of the added bacteria) × 100. A relative percentage of adhesion to or invasion of the mutant and complemented strains was subsequently reported as: (% adhesion to or invasion of mutant or complemented strain/% adhesion to or invasion of the wild-type) × 100. All

experiments were performed in independent triplicate biological experiments with triplicate technical repeats. Data were analyzed by one-way Analysis of Variance (ANOVA), with the statistical analysis performed using SPSS 25.0 statistical software, and a *p*-value less than 0.05 considered significant.

Results:

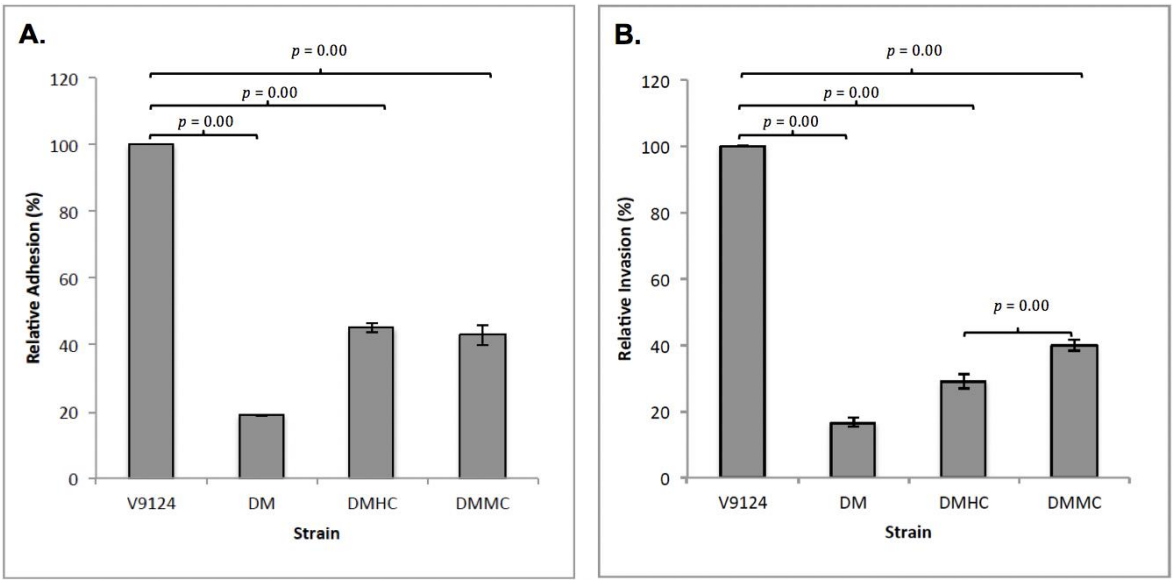

Fig. 1. The role of *M. tuberculosis* heparin-binding haemagglutinin adhesin (HBHA) and curli pili protein (MTP) in the adhesion to (A) and invasion of (B) A549 pulmonary epithelial cells. Cells were exposed to V9124 wild-type, Δ*hbhA-mtp* double mutant (ΔDM) and complemented strains (DMHC – double mutant *hbhA* complement; DMMC – double mutant *mtp* complement) for 1 hour (adhesion assay) and 2 hours (invasion assay) at 37°C. Results are depicted as the percentage adhesion or invasion of the mutant and complemented strain, relative to V9124 as the control. Error bars indicate the calculated ±SEM relative to the calculated mean of 3 biological and triplicate technical repeat experiments. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 25.0 statistical software (Adhesion assay - $F(3,8) = 370.82$, $p < 0.00$; Invasion assay - $F(5,12) = 859.36$, $p < 0.00$).

The ability of the Δ*hbhA-mtp* double mutant strain (ΔDM) to adhere to A549 pulmonary epithelial cells was significantly reduced by 81 % relative to the wild-type strain V9124 ($p = 0.00$) (Fig. 1A). The invasive ability of the mutant was also reduced by 83 % ($p = 0.00$) (Fig. 1B).

Adhesion and invasion rates were partially restored by complementation of the individual genes independently with the *pMV261* expression vector containing either an *mtp* or *hbhA* whole gene insert (Govender et al., unpublished). Complementation with plasmid *pMV261-hbhA* resulted in a phenotype of partially restored adhesion

capacity to 45 % and invasion capacity to 29 % relative to the wildtype strain (Fig. 1 A and B). Complementation with plasmid *pMV261-mtp* restored *mtp* expression and resulted in partially restored adhesion to 43 % and invasion capacity restored to 40 % relative to the V9124 wildtype strain (Fig. 1A and B). However, even though the adhesion and invasion capacity of both the complemented mutant strains remained significantly less than the wildtype ($p = 0.00$), there was no significant difference in the adhesion capacity of the *hbhA*-complemented mutant when compared to the *mtp*-complemented mutant ($p = 0.855$), while the invasion capacity between the two strains differed significantly ($p = 0.00$).

Discussion:

Infection by *M. tuberculosis* is regulated by a range of factors, and involves complex interaction between the host immune system and the bacilli. The mechanism of bacterial adherence to its target tissues is considered as fundamental to the infectious process (Finlay & Falkow, 1997). To establish an active infection, bacteria express adhesins (specialized surface proteins) that mediate adhesion to the host cells and prevent clearance (Niemann et al., 2004). Mycobacterial adhesins that engage with pulmonary epithelial cells may play a fundamental role in the infection process, due largely to these cells most likely being involved in the initial interaction with the host (Pethe et al., 2001). Identifying and characterizing key adhesin molecules involved in the initial attachment to host cells may provide novel targets for drug development and potential new vaccine strategies able to effectively interrupt early host-pathogen interactions.

There is increasing evidence of the significant importance of two well characterised, major adhesins of *M.tb* on interactions with host cells. Actively multiplying *M.tb* bacilli in the lung tissues upregulated *hbhA* expression, prior to the cellular immune response effectively containing the infected areas (Delogu et al., 2006). HBHA was confirmed as a mediator of host-pathogen interactions and an adherence factor, by inhibiting HBHA-mediated hemagglutination and attachment of bacilli to epithelial cells using monoclonal antibodies against HBHA (Menozzi et al., 1996). Pethe et al. (2001)

reported a HBHA-deficient strain to show a reduction in the adhesion to and invasion of A549 pulmonary cells by 60% and 80% respectively, relative to the wild-type strain.

Alteri et al. (2007) confirmed the presence of aggregative, flexible proteins (with biochemical, functional and morphological properties mirroring that of known bacterial pili) in sera obtained from patients with active tuberculosis, and showed that Rv3312A encodes *M.tb* pili (MTP), a surface protein with adhesive properties. Recent studies elucidated a key role for this MTP protein encoded by Rv3312A (*mtp*) in biofilm production (Ramsugit et al., 2013), and as a novel adhesin and invasin of *M. tuberculosis* in its interaction with THP-1 macrophages (Ramsugit and Pillay 2014) and pulmonary epithelial cells (Ramsugit et al., 2016). Similarily to HBHA, a mutant strain deficient in MTP was significantly reduced in its ability to adhere to and invade A549 cells relative to the wild type strain V9124 by 69 % and 56 % respectively (Ramsugit et al., 2016). Ramsugit et al. (2016) proposed that collectively, the available data on both adhesins would suggest that MTP performs marginally better as an adhesin than HBHA, whilst the latter may perform a more effective role as an invasin.

The current study therefore, attempted to provide insight into the role of HBHA and MTP in concert, in adhesion to and invasion of A549 pulmonary epithelial cells, using a double knockout mutant (ΔDM) strain lacking both the genes. Both adhesins demonstrated a significant role in adhesion and invasion. The restoration of gene function using complementation of each gene separately, suggests that in the F15/LAM4/KZN susceptible strain, MTP may have a greater capacity as an invasin of A549 cells than HBHA. However, HBHA and MTP appear to be similar in their role as adhesins. This may allude to potential compensatory mechanisms being upregulated in the absence of either one of the major adhesins, and adhesion capacity being supplemented in the complemented double mutants by expression of additional primary and moonlighting adhesin molecules, such as GAPDH (*gap*), malate synthase (*glcB*), Cpn60.2 (*groEL2*), Rv3717 (N-acetylmuramoyl-L-alanine amidase), Rv0309 (L,D-transpeptidase) 19 kDa lipoprotein antigen and DnaK (Govender et al., 2014). Collectively, the data suggests that these two proteins independently play major roles in the overall adhesion-invasion capacity of *M.tb* V9124, and in combination, this is significantly increased. Therefore, these two surface-associated structures in combination present an attractive option as powerful targets for potential vaccine and or drug development.

A limitation of the study was the absence of a double complement strain to restore full gene function to the double mutant strain. The single genes were independently complemented in the double mutant in order to establish and understand the individual contribution of each gene to the adhesion and invasion capacity of the V9124 strain, relative to each other and in comparison, to the double mutant strain. This insight would not have been possible if a double complement was used. Future studies involving the fully complemented double mutant alongside the single complemented strains to assess the combined effect of the loss of these genes in regulation of key compensatory pathways need to be explored, in both *in vitro* and *in vivo* models.

The development of the double mutant strain additionally pre-empted the generation of the single mutant strain Δ*hbhA*. The availability of both the *hbhA* and *mtp* single gene deletion mutant strains, as well as the double mutant strain, encourages prospective future studies to directly compare the contribution of HBHA and MTP in adhesion and invasion assays in macrophage and mouse models, as well as global transcriptomics to ascertain host immune responses, and pathogen gene regulation.

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Conflict of interest:

None to declare.

Ethical statement:

Ethical clearance was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (REF: 257/13).

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CHAPTER 5: SYNTHESIS

1. Overview

Studies on the roles of mycobacterial adhesin proteins in pathogenesis and disease dissemination have highlighted their importance as potential targets either for development of drugs, or vaccines or diagnostic tests (Díaz-Silvestre et al., 2002; Kinhikar et al., 2006; Alteri et al., 2007; Ragas et al., 2007; Hickey et al., 2010; Kumar et al., 2013; Ramsugit et al., 2013; Naidoo et al., 2014; Esparza et al., 2015; Ramsugit et al., 2014; Ramsugit et al., 2016).

The current study undertook to establish the importance of the combination of 2 major adhesins of *Mycobacterium tuberculosis*, curli pili (MTP) and the heparin binding haemagglutinin adhesin (HBHA), in pathogenesis. These molecules had previously been shown independently to be potential biomarkers that may be targeted for therapeutic strategies (Naidoo et al., 2014; Maio et al., 2018). A functional genomics approach was used to disrupt the two genes encoding these adhesin proteins, to produce incompletely transcribed proteins with impeded functionality.

In order to establish the effect of a lack of functional MTP and HBHA proteins, a double mutant gene knockout strain, *ΔhbhA-mtp* (ΔDM), deficient in functional gene copies of both *mtp* and *hbhA*, was generated. Additionally, complemented ΔDM strains, each restoring expression of a single gene, were constructed. These strains were used alongside the double mutant and wildtype strains to elucidate MTP and HBHA function in growth and viability, as adherence factors in biofilm formation, as well as in their interaction with host epithelial cells.

2. Literature and background in support of study feasibility

In Chapter 1, the current body of knowledge was reviewed in detail to provide background and insight into the problem at hand, the current status of therapeutics, and methods currently being implemented in research endeavours. The historic evolution and development of *M.tb* to become the debilitating global pathogen, as well as the mechanisms it uses to engage with, infect and evade the host was described and outlined. Overall, a picture was painted that highlighted the staggering nature of the

global epidemic, and the crisis resulting from drug resistance and TB/HIV co-infections in population-dense, low income settings. The deficit of effective drug and vaccine candidates, as well as the lack of available true POC tests that may be applied in high-burden settings was explained. Commentary was provided looking at the current pipelines in terms of drug candidates, vaccines and POC diagnostics in development, showing the ongoing progression toward finding solutions to attack and alleviate the problem from every perspective. Further to this, the rationale behind the choice of the target molecules in the study was established, with a basic introduction on their importance in TB pathogenesis.

Chapter 2 (Govender et al., 2014) presented a more detailed review on the adhesin family of molecules. This served to further discuss the role of adhesins in the pathogenicity of *Mycobacterium tuberculosis*, whilst providing insight into structural and functional characteristics of both well-established and putative novel adhesins of the pathogen. Additionally, thorough rationale and insights were provided in support of the potential application of a range of *M.tb* adhesin molecules as valuable and successful targets for the development of TB therapeutics. Key messages include the support for the use of the lesser-studied host cell of *M.tb*, the alveolar pulmonary epithelial cell as an experimental model, as well as an explanation of how mycobacterial adhesins engage with host cells to perpetuate infection. Another key message, was discussion around "moonlighting adhesins" – these include the chaperonin protein encoded by *cpn60.2*, the glyoxylate shunt enzyme malate synthase, the glycolysis protein GAPDH, as well as the heparin-binding haemagglutinin adhesin, HBHA. These proteins were earmarked to be given serious consideration for further development in strategic infection control strategies.

Cumulatively, these initial chapters presented knowledge to allow for a deeper understanding of the impact of the pathogen on the world population, and the challenges encountered in reducing the infection burden. They offer insights on potential targets for therapeutic development, and strong motivating foundation and rationale for the research questions that were addressed in the study presented in this thesis.

3. Generating mutants: genetic manipulation of V9124 to construct mutant and complemented strains to assess gene function

As a preliminary basis of the study, and in order for the experimental assays to be performed, a number of molecular techniques needed to be completed to allow for the construction and molecular confirmations of the model organisms. The wildtype, control organism, *M. tuberculosis* V9124 (a drug-susceptible strain belonging to the F15/LAM4/KZN family) was subject to genetic manipulation using the technique of specialized transduction with phasmids containing allelic exchange substrates to construct mutant and complemented strains to assess gene function.

Initially, a single mutant, Δ*hbhA*, was generated using a phage carrying an allelic exchange substrate targeting ~80% of the *hbhA* gene. Thereafter, a second phage carrying a γδ-resolvase gene was used to perform specialized transduction on a PCR and Sanger sequencing confirmed Δ*hbhA* single mutant. The phage served to remove the resistance gene portion (*hygB-sacB*) of the *hbhA* AES inserted in the native gene within the genome that was used to disrupt gene expression. The resolvase acted on the γδ-resolvase sites flanking the *hygB-sacB* cassette to unmark the Δ*hbhA* single mutant. The unmarked Δ*hbhA* single mutant was confirmed by PCR and Sanger sequencing. This was used as a parent strain to generate the Δ*hbhA-mtp* double mutant strain, as described above, using a phage carrying an allelic exchange substrate targeting ~80% of the *mtp* gene. The double mutant strain Δ*hbhA-mtp* was confirmed by PCR and Sanger sequencing, before being used as the parent strain for complementation.

Complementation was performed with individual genes to produce single complemented strains, Δ*hbhA-mtp-hbha* comp and Δ*hbhA-mtp-mtp* comp. The *hbhA*-complementation plasmid *pMV261-hbhA* was generated by ligating the purified whole wildtype *hbhA* gene into the *pMV261* episomal plasmid backbone with a kanamycin selective gene. The plasmid was confirmed by restriction digest, PCR and Sanger sequencing prior to being electroporated into the Δ*hbhA-mtp* double mutant strain for complementation with *hbhA*. Positive transformants were screened and confirmed by PCR and Sanger sequencing.

Similarily, the *pMV261-mtp* plasmid (harvested from a confirmed Δ*mtp* single mutant by CTAB gDNA extraction followed by plasmid extraction and purification) was

electroporated into the Δ*hbhA-mtp* double mutant strain for complementation with *mtp*. Positive transformants were screened and confirmed by PCR and Sanger sequencing.

These strains were then used to carry out the subsequent downstream experiments including growth assays by OD_{600nm} readings and CFU counts, viability assay by means of the resazurin microplate assay, biofilm quantification by means of crystal violet staining and the adhesion and invasion assay on A549 pulmonary epithelial cells.

4. HBHA and MTP facilitate growth, but not viability and biofilm formation

To determine the dual contribution of the HBHA and MTP proteins to the growth, viability and biofilm formation in the F15/LAM4/KZN drug-sensitive strain V9124, the double mutant strain *ΔhbhA-mtp*, generated by phage-mediated mycobacterial specialized transduction, as described above, was subjected to the growth, viability and biofilm assays alongside the two single-gene complemented and wildtype strains.

The growth assays, by means of OD_{600nm} quantification and CFU counts indicated that HBHA and MTP in combination have a significant effect on the *in vitro* growth of *M.tb*. The growth of the independently complemented double mutant strains suggested that the contribution of each adhesin was approximately equal. Even though the growth rate was significantly impaired, the double mutant strain remained metabolically viable relative to the other strains, thus, suggesting that HBHA and MTP do not contribute to the metabolic activity of *M.tb*.

The concurrent removal of *hbhA* and *mtp* resulted in a non-significant 8.82% reduction ($p > 0.05$) in biomass in comparison to the wild type V9124. The ΔDM *hbhA* complemented strain (*hbhA* expression restored) showed a significant reduction in biomass, and when compared to the lower, insignificant reduction noted in the ΔDM *mtp* complemented strain (*mtp* expression restored). This suggests that MTP may have a greater effect on biofilm formation than HBHA, a finding supported by Ramsugit et al. (2013).

In generating the double mutant and complemented strains, the study was able to illustrate a potential compounded effect on bacterial phenotype, including growth,

viability and biofilm formation. This work supports the postulate that the two major *M.tb* adhesins promote *in vitro* growth and biomass formation in the F15/LAM4/KZN susceptible strain, and thus contribute to the pathogenicity of this strain. These results suggest the importance of the two adhesins and their potential value when targeted in combination for the development of therapeutic interventions.

5. A loss of *hbha* and *mtp* gene expression collectively reduces adhesion and invasion capacity of *M. tuberculosis*

The combined effect of HBHA and MTP in the adhesion to and invasion of host cells, was established in an *in vitro* tissue culture infection model with A549 pulmonary epithelial cells. Significant reduction was shown in adherence to (81%) and invasion of (83%) A549 pulmonary epithelial cells by Δ*hbhA-mtp* double mutant strain (ΔDM), relative to V9124 (p = 0.00). Complementation with *hbhA* partially restored adhesion and invasion to 45% and 29% respectively, while complementation with *mtp* partially restored adhesion and invasion to 43% and 40% respectively, relative to the V9124 wildtype.

Collectively, the data suggests that these two proteins independently play major roles in the overall adhesion-invasion capacity, with MTP having a potentially greater invasin role than HBHA in the *M.tb* F15/LAM4/KZN susceptible strain V9124. However, in combination, invasion capacity is significantly increased. HBHA and MTP appear to be similar in their role as adhesins. Therefore, these two surface-associated structures in combination play a significant role in the overall adhesion-invasion capacity of *M.tb*, and present an exciting option as powerful combined targets for therapeutic drug and vaccine development.

6. General Discussion

Anti-adhesion therapies, although conceptually appealing, remain under-developed and un-utilized in the treatment of bacterial infections, with little to no targeted blocking antibodies currently being investigated for vaccine development. This hesitance is predominantly due to the expression of multiple adhesion molecules occurring in a well-modulated tissue- and time-specific manner from initial contact throughout the course of an infection (Stones and Krachler, 2015). Despite relatively small genomes,

mycobacteria possess a host of molecules that have suspected moonlighting function as adhesins outside of their core functions. These are able to circumvent the effects of a loss of expression of key genes by initiating cascades of compensatory mechanisms, using these moonlighting molecules as back-up adhesins (Neyrolles et al., 2001; Kinhikar et al., 2006; Kumar et al, 2013; Govender et al., 2014). It is most likely that *M.tb* possesses unidentified genes encoding novel factors that hold roles in host cell adherence. Indeed, a major portion of the Mycobacterial genome encodes hypothetical proteins that have yet to be studied and functionally characterized (Mazandu & Mulder, 2012; Kumar et al, 2013; Mann et al., 2016). This challenging issue of redundancy and functional duplication, a potential confounding factor in gene knockout studies, is further highlighted by the presence of the large number of predicted adhesins (as reviewed by Govender et al., 2014).

The approach applied in this study highlights the usefulness of targeting multiple adhesin or moonlighting proteins as a way to effectively mitigate this pitfall in adhesin-directed vaccine development, as the study outcomes may allude to potential compensatory mechanisms being up-regulated in the absence of either one of the major adhesins, and adhesion capacity being supplemented in the complemented double mutants by expression of additional primary and moonlighting adhesin molecules.

A limitation of this study design was the independent complementation of the double mutant strain by the individual genes in separate episomal expression plasmids to generate hypothetical "single mutant strains". This resulted in partial complementation of either *hbhA* or *mtp* in the ΔDM to fully restore only single gene function, and the absence of a truly double complement strain to restore full gene function to the double mutant.

The motivation for generating this type of individual single complemented ΔDM strains was to attempt to assess and establish the individual contribution of each adhesion molecule in the assays performed. In doing this, it was hoped that differences could be detected to allow us to establish if one gene had variable or increased effects on growth, viability and biofilm formation over the other. In this way, a greater understanding of the individual contribution of each gene to the adhesion and invasion capacity of the V9124 strain, relative to each other, and (in comparison) to the double mutant strain,

could be gathered. This insight would not have been possible if a double complement was used.

7. Conclusions

The findings of the present study illustrate the limited effects of a lack of both these genes on bacterial phenotypes, including viability and biofilm formation, while a significant compounded effect is recognized in terms of attenuated growth capacity. Interestingly, MTP appears to have a greater impact as an invasin of A549 pulmonary epithelial cells than HBHA, while both HBHA and MTP appear to be similar in their role as adhesins in this cell type.

These outcomes support the postulate that the two major *M.tb* adhesins promote *in vitro* growth (and biomass formation less significantly) in the F15/LAM4/KZN susceptible strain, while playing key roles in the adhesion and invasion capacity of *M.tb*, thus contributing to the overall pathogenicity of the organism. These two surface-associated molecules, in combination, present an appealing option as powerful targets for potential vaccine and or drug development. This study would then hopefully serve to indicate the value of investigating reducing the functionality of both the major adhesin proteins by potential targeted blocking antibodies, in order to prevent bacterial entry into the host cells, thereby working toward reducing bacterial proliferation and evasion.

8. Future work

To gain a comprehensive and detailed understanding of the combined effect of these two key adhesin-expressing genes acting in concert, further studies utilizing a double-complemented *hbhA-mtp* mutant strain are needed as supplementary steps to substantiate and truly validate the outcomes of this study. Such a complemented strain would enable the full restoration of gene function when both genes are complemented.

Further studies looking into the impact of the absence of these genes on signaling pathways and gene regulation networks (both in *M.tb* and the host) would shed more light into the role they play in facilitating bacterial proliferation, and the potential effect that they may have on host immune modulation.

Studies utilizing the fully complemented double mutant alongside the single complements to assess the combined effect of the loss of these genes in regulation of key compensatory pathways need to be explored, both *in vitro* and *in vivo*. They may also serve to indicate and highlight roles of other potential compensatory genes to be considered as novel therapeutic targets for development alongside *hbhA* and *mtp*. The activity of these supplemental genes with moonlighting ability may come to the surface and indicate if they may be directly impacted by gene expression or repression of either *hbhA* or *mtp*.

The development of the double mutant strain additionally necessitated and pre-empted the generation of the single mutant strain, Δ*hbhA*. The availability of both the *hbhA* and *mtp* single gene deletion mutant strains, encourages prospective future studies alongside the double mutant strain to directly compare the contribution of HBHA and MTP in adhesion and invasion assays in macrophage and mouse models, as well as transcriptomic-based investigations to ascertain host immune responses, and pathogen gene regulation processes.

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Appendix 1 – Supplementary images:

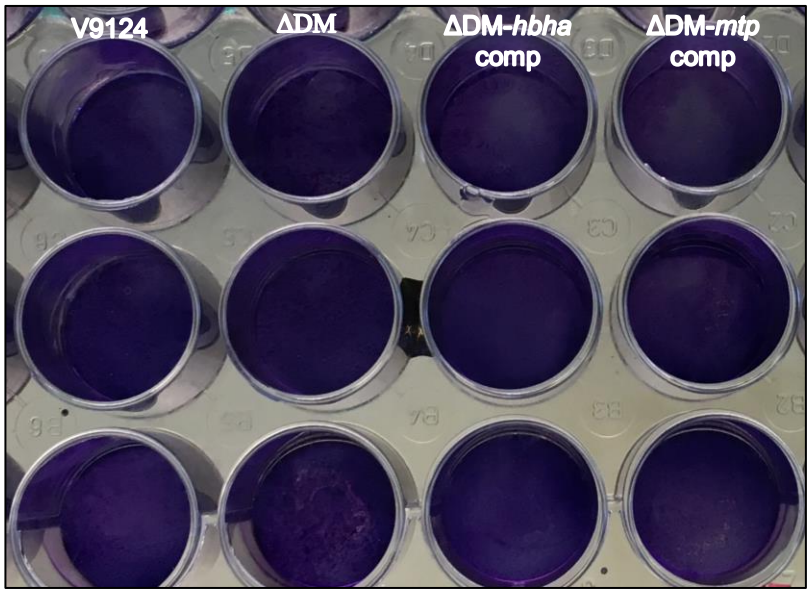

Fig. 1: Biofilm assay - no visible difference in biofilm formation is noted between the wildtype, double mutant and complemented strains, after a 5 week incubation, with blank culture media as a negative control.

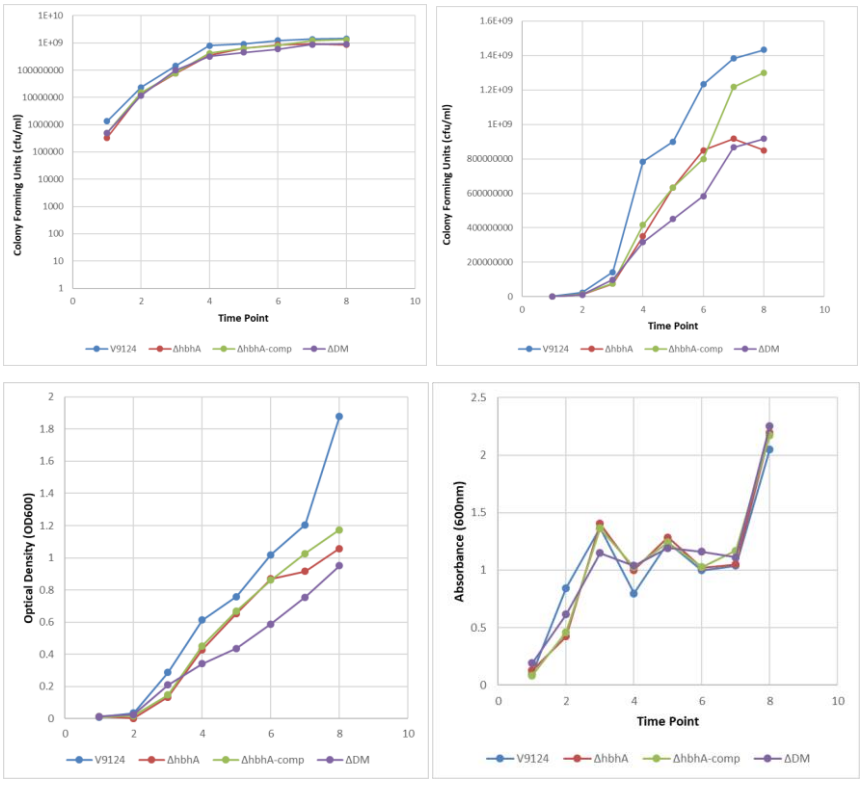

Fig. 2: Growth and viability assay data highlighting the *hbhA* single mutant and complemented *hbhA* single mutant strain, relative to V9124 and ΔDM.

Appendix 2 - Supplementary tables:

Chapter 3 – Paper 2

Table 1: Growth Assay – Mean Optical Density (600nm)*

Timepoint	**V9124**	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Day 0	0,01	0,02	0,03	0,01
Day 3	0,04	0,03	0,00	0,01
Day 6	0,29	0,21	0,16	0,13
Day 9	0,61	0,34	0,45	0,43
Day 12	0,76	0,44	0,64	0,60
Day 15	1,02	0,59	0,80	0,81
Day 18	1,20	0,76	0,91	0,92
Day 21	1,88	0,95	1,11	1,11

Table 2: Growth Assay – Mean Colony forming units per mL (CFU/mL)*

Timepoint	**V9124**	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Day 0	1,33E+06	5,00E+05	5,00E+05	5,00E+05
Day 3	2,33E+07	1,17E+07	1,83E+07	2,50E+07
Day 6	1,43E+08	9,83E+07	7,67E+07	4,67E+07
Day 9	7,83E+08	3,17E+08	3,50E+08	3,17E+08
Day 12	9,00E+08	4,50E+08	5,67E+08	5,50E+08
Day 15	1,23E+09	5,83E+08	6,83E+08	6,50E+08
Day 18	1,38E+09	8,67E+08	9,17E+08	8,67E+08
Day 21	1,43E+09	9,17E+08	1,15E+09	1,12E+09

Table 3: Resazurin Microplate Viability Assay – Mean Absorbance (600nm)*

Timepoint	**V9124**	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Day 0	9,55E-02	1,90E-01	2,02E-01	1,24E-01
Day 3	8,43E-01	6,14E-01	5,25E-01	4,77E-01
Day 6	1,37E+00	1,15E+00	1,48E+00	1,56E+00
Day 9	7,96E-01	1,04E+00	1,04E+00	8,33E-01
Day 12	1,24E+00	1,19E+00	1,24E+00	1,32E+00
Day 15	9,97E-01	1,16E+00	9,91E-01	9,93E-01
Day 18	1,04E+00	1,11E+00	1,13E+00	1,03E+00
Day 21	2,05E+00	2,25E+00	2,17E+00	2,21E+00

Table 4: Biofilm Assay – Absorbance 600nm*

	V9124	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Mean Run 1	0,61	0,53	0,55	0,56
Mean Run 2	0,55	0,53	0,49	0,53
Mean Run 3	0,58	0,53	0,52	0,54
Mean	0,58	0,53	0,52	0,54
Std. Dev	0,02	0,00	0,02	0,01
Relative %	100,00	91,18	90,70	94,40

Note: Data presented are overall means of three biological repeats within three technical repeats

Chapter 4 – Paper 3

Table 5: Adhesion Assay – % Adhesion

	V9124	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Mean Run 1	0,25	0,05	0,12	0,11
Mean Run 2	0,27	0,05	0,12	0,10
Mean Run 3	0,21	0,04	0,09	0,10
Mean	0,24	0,05	0,11	0,10
Std. Dev	0,0193	0,0035	0,0110	0,0014

Table 6: Adhesion Assay – % Relative Adhesion

	V9124	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Mean Run 1	100,00	19,35	48,00	44,00
Mean Run 2	100,00	18,52	44,44	37,04
Mean Run 3	100,00	19,05	42,86	47,62
Mean	100,00	18,97	45,10	42,89
Std. Dev	0	0	2	3

Table 7: Invasion Assay – % Invasion

	V9124	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Mean Run 1	0,25	0,04	0,07	0,10
Mean Run 2	0,27	0,04	0,07	0,10
Mean Run 3	0,21	0,04	0,07	0,09
Mean	0,24	0,04	0,07	0,09
Std. Dev	0,0153	0,0011	0,0013	0,0021

Table 8: Invasion Assay – % Relative Invasion

	V9124	**ΔDM**	**ΔDM-***hbhA* comp	**ΔDM-***mtp* comp
Mean Run 1	100	16,00	28,00	40,00
Mean Run 2	100	15	26	37
Mean Run 3	100	19	33	43
Mean	100	17	29	40
Std. Dev	0	1	2	2

Appendix 3 – Biomedical Research Ethics Committee (BREC) Approval Letters – Full Study Approval & Recertification:

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 November 2013

Ms VS Govender
719 Umbilo Road
Congella
4013
shalomgovender@gmail.com

PROTOCOL: The role of the anchorless adhesion malate synthase in the pathogenesis on Mycobacterium tuberculosis. REF: BE257/13.

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 11 July 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 30 October 2013 to queries raised on 12 September 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 11 November 2013. **Please note that MTA and Export Permit are required by BREC before any shipping of samples.**

This approval is valid for one year from **11 November 2013.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **10 December 2013.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

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14 November 2018

Ms VS Govender
719 Umbilo Road
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Dear Ms Govender

PROTOCOL: The in vitro roles of the major adhesins HBHA and MTP in the pathogenesis of M.tuberculosis, using a novel double gene knock-out mutant strain generated by specialised transduction. REF: BE257/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	11 November 2018
Expiration of Ethical Approval:	10 November 2019

I wish to advise you that your application for Recertification received on 08 November 2018 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 December 2018.

Yours sincerely

PK

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc: pillay2@ukzn.ac.za

Appendix 4 – Turnitin Report:

FINAL Thesis November 28 2018_From Chapter 1 to End_No Prelim_No references_NO Chapter 2 Published Review_Turnitin

by Viveshree Govender

Submission date: 28-Nov-2018 09:55PM (UTC+0200)
Submission ID: 1046158956
File name: Prelim_No_references_NO_Chapter_2_Published_Review_Turnitin.pdf (3.52M)
Word count: 24223
Character count: 134246

FINAL Thesis November 28 2018_From Chapter 1 to End_No Prelim_No references_NO Chapter 2 Published Review_Turnitin

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