Characterization of the function of RV1268c, an ATP binding cassette transporter in *Mycobacterium tuberculosis.*

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Dissertation Submitted in fulfilment of the requirements for the Degree of Master's in Medical Microbiology

SCHOOL OF LABORATORY SCIENCE, MEDICINE, AND MEDICAL SCIENCES
DEPARTMENT OF MEDICAL MICROBIOLOGY

February 2023

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DECLARATION

I, Pumelela Hallom declare that this dissertation, submitted to the University of KwaZulu Natal for the degree of Master's in Medical Microbiology in the school of laboratory Science, Medicine, and Medical sciences is my own work and has never been submitted to any other university for the award of any degree.

I declare that I followed the rules with regards to referencing and citation in scientific writing.

Name: Pumelela Hallom

Signature…… 

Date……30/03/2023

DEDICATION

This dissertation is dedicated to the memory my late mother Thembisa Hallom. I am grateful for her consistent love and for being my emotional anchor through my whole existence. She walked with me throughout this project without hesitating any moment of seeing me finishing this project until her demise.

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my supervisor, Dr Sibusiso Senzani for his persistent guidance and supervision throughout the duration of this study. His mentoring and constructive criticism has been pivotal in the advancement of my scientific knowledge.

I would like to thank the University of KwaZulu Natal for giving me the opportunity to further my studies in their institution. To the South African National Research Foundation for the financial support, allowing me to be able to enrol for this degree.

To my late mother, I am grateful for her support and prayers as I believe they always carried me through. My fiancé (Akhona Xuma) for being my strength when I felt weak, and continuous support. My little sister (Nikiwe) for endless love and support and my 2 kids (Mivuyo and Langa) for being my motivation and sources of laughter in sad moments.

I would like to send my appreciation to my entire family and friends for their prayers and support. A shoutout to my friends Khumbuzile and Palesa whom we referred to ourselves as KP^2, for their unending support.

Lastly, I would like to thank God for the strength and courage he gave me to continue pursuing this study after a lot of thoughts of quitting when I felt like my life had come to an end after losing my mom.

ABSTRACT

Tuberculosis (TB) is an epidemic disease that is caused by a bacterium called *Mycobacterium tuberculosis* (*Mtb*). This disease infects and kills millions of people globally. Anti-Tuberculosis (anti-TB) drugs such as isoniazid, ethambutol, pyrazinamide, and fluoroquinolones have been discovered and produced for TB treatment but regardless, TB persists because of the resistance to these drugs, leading to the development of multidrug-resistant (MDR) *Mtb* and extensively-drug resistant (XDR) *Mtb*. One of the key areas to focus on for the development of new effective anti-TB drugs are efflux systems, because they transport molecules outside cells and have a role in the resistance against TB treatment.

This study aimed to identify the biological function of the *Mtb* protein, Rv1268c. RV1268c was one of the identified proteins in a study that was done by Chiliza *et al., 2019* where a few protein biomarkers that are recognized by both active TB and latent TB patient antibodies. Some of these biomarkers that were studied are TreY, Bfr, and TrpG, which are biomarkers that are specific to ATP and play an important role in pathogenesis. Other biomarkers included MoaE, PonA1, and NarG, which are specific to latent TB and play a role in dormancy.

The Rv1268c protein of *Mtb* is classified as a hypothetical membrane protein of the cell envelope and its associated proteins are Rv1267c and RV1269c, which are regulatory protein and a conserved putatively exported protein, respectively. The Rv1268c protein is hypothesised to be an ATP-binding cassette (ABC) transporter. The nucleotide and protein sequences of Rv1268c were

downloaded from the database of *Mtb* H37Rv using Mycobrowser. To create a knock down strain, annealed oligos were ligated to PLJR965 plasmid and transformed into XL10-Gold ultracompetent cells and grown on kanamycin-containing plates. Extracted plasmids were electroporated into *Mtb*, and after 4 weeks of incubation, the colonies were screened to check if they carried the knock down plasmid . DNA was then extracted to characterize the function of the Rv1268c *Mtb* protein. The results showed that Rv1268c had no effect on the in vitro growth of *Mtb*, while the Ethidium bromide (EtBr) assay displayed a difference on the extrusion of EtBr as the knock down and the knock down with anhydrotetracycline (aTc) had lower fluorescence as compared to the wild type which implies that Rv1268c is not an ABC transporter. The statistical analysis showed that the was no significant difference on the drug susceptibility between the wild type, knock down, and the knock down with aTc strains . The growth of neither the wild type or knock down strains was completely inhibited by either of the drugs tested.

Keywords: *Mycobacterium tuberculosis*, multi-drug resistant, extensively-drug resistant, Rv1268c, ABC transporter

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NOMENCLATURE

µg	: Microgram
ABC transporter	: ATP-Binding cassette transporter
AIDS	: Acquired Immunodeficiency Syndrome
aTc	: Anhydrotetracycline
ATP	: Adenosine trisphosphate
bp	: Base pairs
DNA	: Deoxyribonucleic acid
CRISPRi	: Clustered regulatory interspaced short palindromic repeats
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
EDTA	: Ethylenediaminetetraacetic acid
EtBr	: Ethidium bromide
HIV	: Human I immunodeficiency Virus
Kan	: Kanamycin
KanR	: Kanamycin Resistance
Kb	: Kilo base pair
L	: Litre
LTBI	: Latent tuberculosis infection
MDR	: Multi-Drug resistant
MDR-TB	: Multidrug-Resistant Tuberculosis
$MgCl_2$	: Magnesium chloride
$MgSO_4$	: Magnesium sulphate
MIC	: Minimum inhibitory concentration
Min	: minutes
Ml	: Millilitre
Mtb	: Mycobacterium tuberculosis
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NBD	: Nucleotide binding domains

OADC	: Oleic Albumin Dextrose catalase
OD600	: Optical density at 600 nanometer wavelengths
PAM	: Protospacer adjacent motif (PAM)
PCR	: Polymerase Chain Reaction
R	: Resistant
RT	: Reverse Transcriptase
sdH2O	: Sterile distilled water
TB	: Tuberculosis
TMDS	: Transmembrane domain:
Tris	: Trisaminomethane
TST	: Tuberculin Skin test
Tween	: Polyoxyethylene sorbitan monooleate
WHO	: World Health Organization
WT	: Wild type
XDR	: Extensively-Drug resistant
XDR-TB	: Extensively Drug Resistant Tuberculosis

CHAPTER ONE

1.1 History of Tuberculosis

Tuberculosis (TB), a disease that is caused by *Mycobacterium tuberculosis* (*Mtb*), is one of the most persistent and deadly diseases globally (WHO, 2018). TB remains a major health concern because of high mortality rates associated with this disease (WHO, 2018). Annually, more than 1.3 million mortality cases have been reported due to this disease worldwide (WHO, 2018). Evidence of *Mtb* in Neolithic remains and Egyptian mummies dates back to 2400 BC (Morse *et al*., 1964; Zimmerman and Bull, 1979), where studies have shown that *Mtb* was hypothesised to have originated from *Mycobacterium bovis* and transmitted to humans through the domestication of cattle (Morse, 1961; Hare, 1967). Over the centuries, scientists have tried to research and understand where TB came from. French physician Jean-Antoine Villemin demonstrated that TB is an infectious disease and was transmitted by human contact to animals and transmission from one animal to another (Koch, 1882). Villemin infected rabbits with TB obtained from a dead patient that died because of TB infection (Koch, 1882). However, Villemin's findings were not considered until other scientists corroborated his findings years later (Koch, 1882).

In 1882, Robert Koch first identified *Mtb* (Daniel, 2006) and determined that TB was caused by *Mtb* (Harding, 2010), and he was the first to cultivate *Mtb* (Daniel, 2006). Robert Koch described his postulates, known as Koch's postulates, which are parameters set to follow when identifying the causative agent of infectious diseases (Daniel, 2006; Gradman, 2006). Following the understanding of TB's origin, in 1890, Koch discovered a possible vaccine against TB by isolating a substance that he called tubulin (Daniel, 2006). Still unfortunately, it was not effective as a vaccine, because in a medical trial that was performed, tuberculin failed to provide a protective action in patients to whom tuberculin was administered as only 1% of patients in the trial were

cured (Huebner *et al*., 1993), then Koch initiated the tuberculin skin test (TST), believing that tuberculin could be used to detect TB (Daniel, 2006). Years later, a vaccine against TB was invented by physician Albert Calmette and veterinarian Camille Guérin with the use of *Mycobacterium bovis* (*M. bovis*), an organism isolated from tuberculous cattle (Daniel and Léon, 2005). However, in their cultured strains of *M.bovis*, they could not create a homogenous suspension (Daniel and Leon, 2005). Upon the addition of ox bile to prevent the formation of the clumps, virulence in guinea pigs infected with *M. bovis* was reduced (Daniel and Leon, 2005). The Bacillus Calmette Guérin (BCG) vaccine was first used in humans when administered to a 3 days old infant whose mother had died due to TB after giving birth, and no undesirable outcome was achieved (Calmette, 1928).

1.2 *Mycobacterium tuberculosis* infection

Mtb infection is initiated when bacilli entailed in droplets from a person who has active TB are inhaled; the droplets are transmitted through coughing or sneezing (McShane, 2005). Upon the arrival of bacilli droplets in the lungs, the innate immune response is activated where the *Mtb* is engulfed by macrophages (MA), that most often kill the entering bacteria (Cooper, 2009), or dendritic cells within the lungs (Modlin and Bloom, 2013). At this stage, the bacilli are either eradicated or retained in the macrophages (Russell *et al*., 2010) because *Mtb* can evade the host's immunity by triggering the anti-inflammatory response, blocking the production reactive oxygen and nitrogen intermediates, or reducing the acidification of the phagosome that contains the *Mtb* (Russell *et al*., 2010).

If *Mtb* escapes the innate immune response, it starts to replicate within the macrophages (Wolf *et al*., 2007). Then other cells such as monocytes, neutrophils, and lymphocytes are recruited from blood vessels to the infection site (Davis and Ramakrishnan, 2009). This subsequently results in the formation of granuloma, which are cell aggregates that are the pathological feature of TB (Davis and Ramakrishnan, 2009). This stage of TB is regarded as latent TB because *Mtb* cannot spread as it is contained within the granuloma latent (Russell *et al*., 2010). When the TB-infected host is immunocompromised by conditions such as co-infection with HIV, which affects CD4 T cells which have a pivotal role in the control of *Mtb*, the granuloma breaks, releasing the caseum (Russell, 2007; Russell *et al*., 2010) which is a soft necrotic detritus that accumulates in the centre of the granulomas (Palaci *et al*., 2007). This results in reactivation of active TB disease, which is transmissible (Russell, 2007; Russell *et al*., 2010).

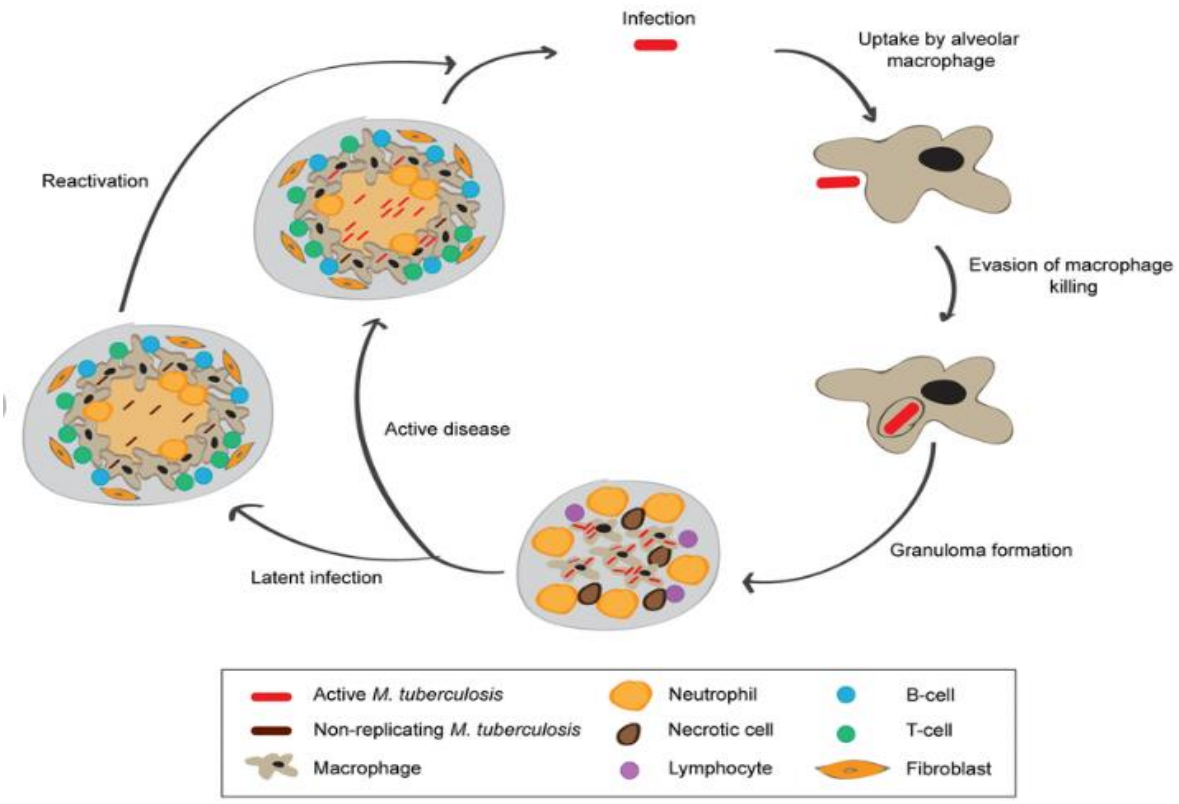

Figure 1.1 *Mtb* infection transmission occurs the droplets that entail bacilli are inhaled. The bacilli travel to the lungs and are engulfed by alveolar macrophages (AM). Within the AM, the bacilli are exposed to the reactive oxygen and nitrogen species that are produced by macrophages. *Mtb* can evade macrophage killing by inhibiting phagosome-lysosome fusion. This results to the mounting of proper immune response and in 90% cases, *Mtb* will be contained, and results in a latent infection. In 10% of cases, the disease progresses and develops to active infection in which *Mtb* is released from the granulomas. Latent infection can be reactivated and develops to active infection. Image from Sawyer *et al*.,2018

1.3 Diagnosis of TB

Improvement in the accuracy and efficiency of TB diagnosis plays a huge role in the success of treatment (WHO, 2021). Culturing *Mtb* in solid or liquid media remains the best diagnostic test (Asmar and Drancourt, 2015). However, this method takes time because *Mtb* grows slowly, taking 4 to 6 weeks in solid media and 7-12 days in liquid media (Mitchison, 2005). Sputum swab microscopy has been a widely used diagnostic test (Lange and Mori, 2010; Newton *et al*., 2008) despite having the lowest sensitivity, where rapid detection of TB for all new cases is 44% and 15-20% of children (Lange and Mori, 2010; Newton *et al*., 2008). Currently, two widely utilised tests are (i) the Mantoux tuberculin skin test (TST) and the Interferon-gamma release assay (IGRA). TST is a skin test that is conducted by injecting tuberculin into the skin (CDC, 2022), and the patient is required to return to the health facility in 48 to 78 hours for the arm that has been injected to be examined whether TB infection could be present or not (CDC, 2016). IGRA is a blood test that evaluates the response after TB proteins have been mixed with a patient's blood (CDC, 2022).

These tests have some limitations because the patient needs to do a follow-up visit for the interpretation of results, and there is a possibility of false-positive results (Mazurek *et al*., 2010). In 2010, the WHO recommended the use of the Xpert MTB/RIF test (WHO, 2011), which utilises a hemi-nested real-time polymerase chain reaction (hnRT-PCR) to amplify *Mtb*-specific sequences (EL-Hajj *et al*., 2001; Piatek *et al*., 1998). The Xpert MTB/RIF test has higher sensitivity for extrapulmonary TB diagnosis and is highly accurate when it comes to detecting rifampicin resistance within 2 hours (Kohli *et al*., 2021).

1.4 Treatment of TB

1.4.1 History of TB treatment

The BCG vaccine that is currently in use was developed using a weakened *M.bovis* strain in the 1930s (Thao and Escalante, 2011). The BCG vaccine has proven to have limited protection with 0% to 80% protective effect because of varying reasons, from the use of varied BCG strains to immune factors (Fine *et al*.,1999). Yet, it remains the only vaccine used (Wang *et al*., 2010). Currently TB treatment relies primarily on antibiotics (Nambi *et al*., 2010). Streptomycin was the first effective anti-TB drug, which was discovered in 1944 (Schatz *et al*., 1944). However, it was later discovered that alone, it was not enough to eradicate *Mtb* (Morrison *et al*., 2009). In 1946, para-aminosalicylic acid (PAS) was discovered (Herzog, 1998). Although Streptomycin and PAS were both effective in treating TB, when used separately as a single agent, they caused drug resistance (Hinshaw *et al*., 1946). This led to the conclusion that a single drug agent should never be used for the treatment of active TB (Robitzek *et al*., 1952).

In 1951, Isoniazid was discovered (Yang *et al*., 1992: Yang *et al*., 1993), and it was the most effective drug against TB, until patients relapsed when treated with isoniazid alone (Robitzek *et al*., 1952). This substantiated the fact that multi-drug treatment was the correct route for chemotherapy (Robitzek *et al*., 1952). Streptomycin, PAS, and Isoniazid were then used as combined therapy (Iseman, 2002) until drug resistance was observed (Thomas *et al*., 1961). Subsequently, in 1960s, PAS was replaced with ethambutol (Doster *et al*., 1973). Ethambutol reduced the treatment duration 18 months (Doster *et al*., 1973). Rifampicin was then introduced, and it was combined with streptomycin, isoniazid, and ethambutol (Hong Kong Chest Service, 1979; Singapore TB Service, 1986). This combination resulted in more effective cures with greater

than 95% of cures in 8-9 months (Hong Kong Chest Service, 1979; Singapore TB Service, 1986). The next step was the inclusion of pyrazinamide, which reduced the duration of treatment to 6 months when combined with isoniazid and rifampicin (Hong Kong Chest Service, 1979; Singapore TB Service, 1986).

1.4.2 Current treatment

Currently, the primary prescribed anti-TB regimens comprise isoniazid, rifampicin, pyrazinamide, and ethambutol (WHO, 2021), with a cure rate greater than 95% (Zumla *et al*., 2013). However, for effective treatment, 6 months of continuous treatment is required (Berrington *et al*., 2007). With this long-term chemotherapy, patients are most likely to stop adhering to treatment (Dheda *et al*.,2013; Horter *et al.*, 2020), resulting in the emergence of resistant strains which compromise the treatment (WHO, 2010). Resistance of *Mtb* to front-line and second-line anti-TB is an ongoing problem that has resulted in the emergence of MDR-*Mtb* and XDR-*Mtb* (WHO, 2010).

New anti-TB drugs that are presently included in the developmental pipeline consist of compounds that inhibit bacterial cell wall synthesis, protein synthesis, and ATP synthesis (WHO, 2013). One of the key areas to focus on for anti-TB discovery are ATP-binding cassette transporters (ABC transporters). This is because *Mtb* uses the mechanism of active efflux to export drugs out of the cells (WHO, 2013). Genome sequencing has demonstrated a few potential drug pumps which are encoded in *Mtb* (WHO, 2013). In a study conducted by Therese *et al*., 2012, four *Mtb* efflux pumps were proven to have a crucial role in the efflux of anti-TB drugs, which substantiates the need to discover and study more new pumps as they cause resistance to anti-TB drugs by pumping the drugs outside the cells (Therese *et al*., 2012).

1.5 The current status of TB

Despite the discovery of first-line anti-TB drugs (isoniazid, ethambutol, pyrazinamide and rifampicin) (Pourakbari *et al*., 2016; Cao *et al*., 2019) and the TB vaccine, *Mtb* still infects millions of people globally, with 1.5 million people losing their lives to TB in 2020 alone (WHO, 2021). *Mtb* infection cases continue to rise (WHO, 2021) because of the emergence of resistant strains to anti-TB drugs, causing Multidrug-resistant (MDR) *Mtb* and Extensively resistant (XDR) *Mtb* (WHO, 2021). Approximately 14 million people are co-infected with Human Immunodeficiency Virus (HIV) and *Mtb* globally (Getahun *et al*., 2010). It is estimated that HIV infection increases the risk of TB 20-fold as compared to HIV negative individuals (WHO, 2013). HIV-infected people are more likely to develop active TB because of the depletion of $CD4^+$ T- cells which results to the loss of immune response to *Mtb* in HIV positive individuals (Geldmacher and Koup, 2012). The decline of the $CD4^+$ T- cell counts also causes an increase of the rate of latent TB reactivation (Geldmacher and Koup, 2012). The prevalence of TB-HIV co-infection varies greatly, TB prevalence among HIV-infected individuals ranged from 2.9 to 64.5%, while HIV prevalence among TB patients ranged from 3.8 to 72.3%. (Gao *et al.*, 2013). HIV-co-infected individuals have an impact on the persistence of TB because they complicate TB treatment (Abramovitch *et al*., 2011) due to the weakened immune system, which can result in *Mtb* infection progressing to TB disease in a short period of time (CDC, 2016).

The first-line anti-TB drugs are a long treatment course that must be administered for 6 months (WHO, 2021). Although these regimens are effective in treating TB, drug resistance still persists

because, without proper support, patients may find it hard to adhere to treatment, because of the duration of treatment (WHO, 2021). Hence new anti-TB drugs that will be effective in a short period of time need to be developed, and this can be done through understanding how *Mtb* survives within the host, and this study focused on this aim to aid in the discovery of new anti-TB drugs.

1.6 ATP-BINDING CASSETTE TRANSPORTERS

For bacterial cells to survive, cellular functions need to be regulated, where the import of nutrients and the export of unwanted substances into and out of cells, respectively, need to take place (Foronda, 2021). In this process, ABC transporters play a crucial role (Dasa, 2011). ABC transporters are membrane proteins that facilitate the translocation of molecules in and out of the cells with the aid of ATP (Davidson *et al*., 2008; Cui and Davidson, 2011). Translocation of organic and inorganic substrates into and out of cells is crucial for homeostasis for all living organisms (Blattner *et al*., 1997). Proteins of the ABC transporter superfamily are present in all living organisms, and they are regarded as one of the broadest classes of transporters (Higgins, 2003). About 2.5% of the *Mtb* genome encodes ABC transporters, and this proves the importance of these transporters in cellular survival (Braibant and Gilot, 2000). A total of 27 ABC transporters found in *Mtb* were reported in the year 2012 (Youm and Saier, 2012).

Based on the direction of translocation of molecules, ABC transporters are characterised as importers, which are only present in bacteria, and exporters, present in both eukaryotes and prokaryotes (Ambudkar *et al*., 2003). Importers are responsible for the uptake of molecules such as amino acids, nutrients, sugars for bacterial growth and survival (Ambudkar *et al*., 2003).

Exporters transport molecules such as toxins, antibiotics out of cell, assisting in cellular survival as well (Ambudkar *et al*., 2003). Since ABC exporters transport antibiotics outside the cell, they are directly associated with the emergence of antimicrobial resistance (Braibant and Gilot, 2000).

1.6.1 Structure and Function of ABC Transporter Components

The first structure of an ATP ABC transporter was published in 2001 by Chang and Roth (Chang and Roth, 2001). Structurally, despite the importer and exporters directionalities, they are both comprised of the same structure, comprised of two transmembrane domains (TMDs), whose function is to serve as the passage for substrate, and two nucleotide-binding domains (NBDs), which function to hydrolyse ATP for the translocation of substrates (Higgins, 2001; Linton, 2007). Even though all ABC transporters are comprised of these four domains, importers possess an extra domain known as the substrate-binding domain (SBP), which functions to recognise and transport the substrate to the TMDs binding site (Phan *et al*., 2002). The four domains (TMDs and NBDs) are either encoded as separated polypeptides or combined into a protein with several domains (Higgins *et al*., 1986).

Translocation of substrates is possible through an alternating-access mechanism (Johnson and Chen, 2017). The change in the NBD structure that is dependent on ATP results in the TMDs structurally conforming to an inward open and outward open position which allows the substrates to bind and be transported through the membrane (Johnson and Chen, 2017).

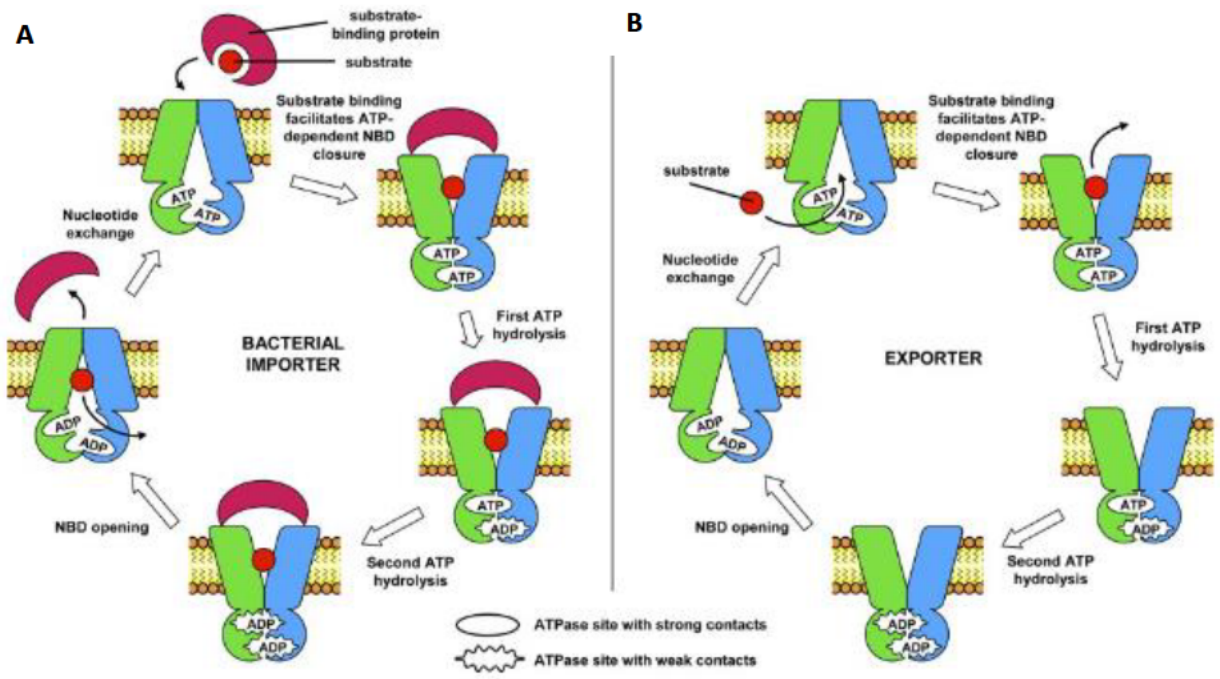

Figure 1.2. A general ABC transporter mechanism for transport of molecules. In the resting state, the NBDs remain open with affinity for ATP. The transport cycle starts when the substrate binds to the high affinity site located on the TMDs, which then induces the conformational change in the NBDs and enhances the binding of ATP. The two ATP molecules bind and result to a closed NBD configuration. The closed NBD triggers a closed conformational change in the TMDs , resulting in opening of the TMDs which forms a chamber opening in the opposite of the initial state. The substrate affinity to the TMDs gets reduced, resulting in the release of the substrate. The ATP hydrolysis follows, and the phosphate (P_i) is released. ADP then restores the transporter to its resting configuration (Davidson *et al.*,2008; Pohl *et al.*, 2005; Rees *et al.*, 2009). Image from Erick *et al.*, 2009

1.7 RV1268c

In a study that was conducted by Chiliza *et al.*, 2019, a number of novel and easily accessible disease specific *Mtb* secreted protein biomarkers were identified. These biomarkers can be used to differentiate active TB (ATB) from latent TB (LTBI) (Chiliza *et al.*, 2019). Some of the identified biomarkers specific to ATB included 4 proteins that are essential for *Mtb* pathogenesis, namely TreY, TrpG, BfrA and Alr (Chiliza *et al.*, 2019). Other proteins specific to LTB that are associated with dormancy included PonA1, PonA2, MoaE1,NarG, and HspR (Chiliza *et al.*, 2019). Among the proteins discovered, Rv1268c was one of the 23 proteins identified to be common to

ATB and LTB (Chiliza *et al*., 2019). Rv1268c is classified as a hypothetical membrane protein of the cell envelope (Song *et al*., 2008). and its associated proteins are Rv1267c and RV1269c, which are regulatory protein (Jang *et al*., 2010) and a protein that is involved in the cell processes (Malen *et al*., 2007), respectively.

1.8 PROBLEM STATEMENT

Mtb is the agent that causes TB, with approximately 10 million people infected in 2018, and in 2019, 1.2 million lives were lost (Dulberger *et al*., 2020). TB treatment is available; however, it takes more than six months to be effective (WHO, 2018; Miglori *et al*.,2007). This can lead to the withdrawal of treatment, and result to the emergence of MDR and XDR strains of *Mtb* (WHO, 2018; Miglori *et al*.,2007). Despite the availability of BCG vaccine for approximately 100 years, the *Mtb* infection rate is still approximately one in three people globally (Zhai *et al*.,2019; Zungisa *et al*.,2015). Thus, it is necessary to determine other drug targets that could be used in the discovery of new anti-TB drugs.

Chiliza *et al* identified biomarkers specific to ATB that are essential for *Mtb* pathogenesis and proteins specific to LTB that are associated with dormancy (Chiliza *et al*., 2019). Among the proteins discovered, Rv1268c was one of 23 proteins identified to be common to ATB and LTB (Chiliza *et al*., 2019). Understanding the functions of important *Mtb* proteins is a priority in identifying and discovering potential new drug targets hence this study aimed to characterize the function of Rv1268c.

1.9 AIMS AND OBJECTIVES

This study aimed to identify the biological function of the *Mtb* gene, Rv1268c, and its involvement in pathogen-host interactions.

OBJECTIVES

- To do *in silico* cloning using Mycrobrowser
- To classify protein families using InterPro
- To study protein-protein interactions using string
- To predict protein structure and function using Phyre2
- To generate a mutant by knocking down Rv1268c
- To characterise the function of Rv1268c, the growth phenotypes of the knock down and the knock down with aTc will be compared to the wild type strain using the following assays: (i) Ethidium bromide assay and (ii) Minimum inhibitory concentration assay.

CHAPTER TWO

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The characterisation of Rv1268c protein was done by constructing a knock down strain utilising CRISPR mechanism. Phenotypic analysis of the protein was done by utilising three assays, namely; growth analysis, ethisium bromide assay and minimum inhibitory concentrations of selected drugs (**Figure 2.1**)

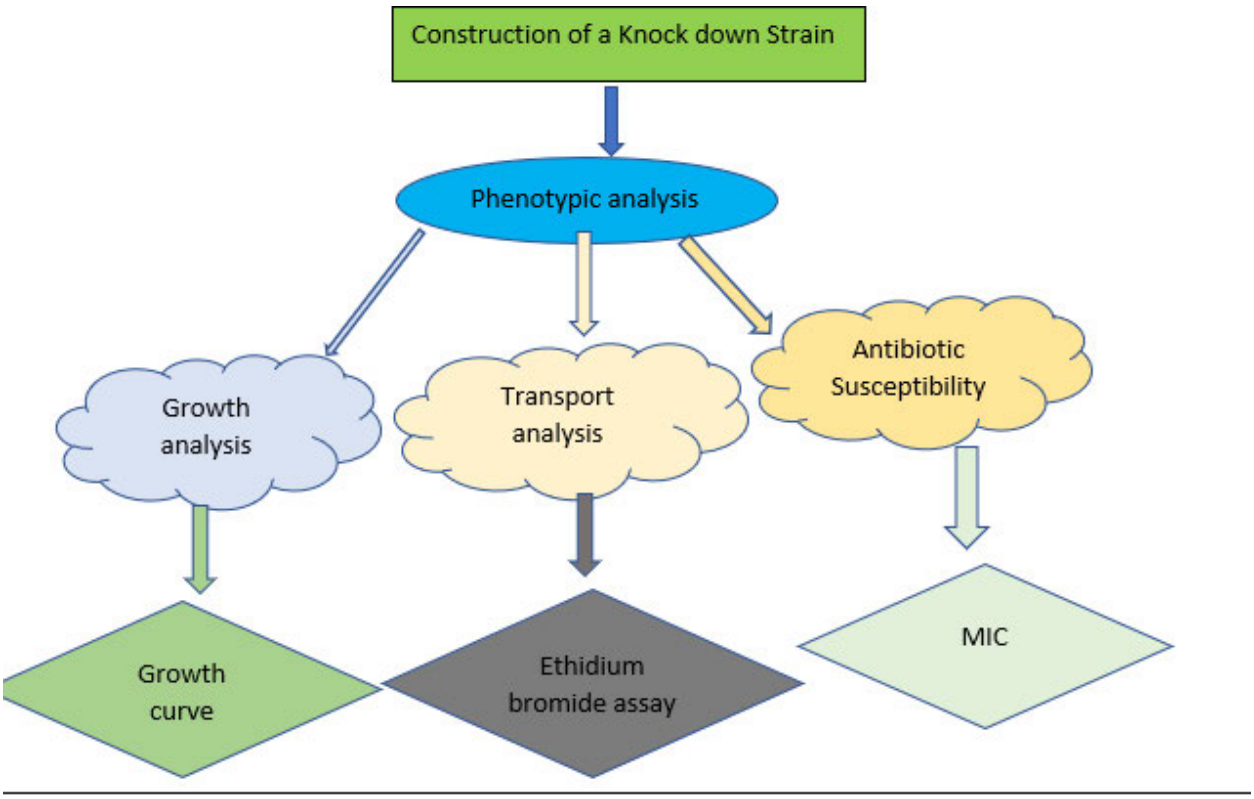


Figure 2.1: Diagramme representing the experimental layout of the study.

2.1 Materials and Methods (Molecular studies)

2.1.1 Ethical clearance

This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00001530/2020).

2.1.2 Bioinformatic analyses

Mycobacterial protein analysis using computational prediction has been a useful approach (Kaspar, 2008). A greater understanding of ABC transporters requires a combination of structural, biochemical, biophysical, and physiological analysis (Locher, 2008). To characterise the function of Rv1268c *in silico*, bioinformatics tools were employed. The *Mtb* Rv1268c DNA and protein sequences were obtained from Mycobrowser (https://mycobrowser.epfl.ch). The DNA sequence was used to do *in silico* cloning. String (https://string-db.org) was utilised to identify potential protein-protein interactions to predict the function of Rv1268c by looking at the functions of the proteins it interacts with. The protein sequence was used for the protein domain prediction, using InterPro (www.ebi.ac.uk/interpro/) to study the combinations of domains for Rv1268c functional analysis. Protein structure determination was done using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) to have a greater understanding of how Rv1268c works by studying the function of the structural homologue that is a close match to Rv1268c.

2.1.3 Bacterial Strains and vectors

The bacterial strains and plasmids utilised in this study are listed in Table 2.1 and Table 2.2 below.

Table 2.1 Bacterial strains that were used in this study

Bacterial strain	Genotype	Reference
Escherichia coli XL10-Gold	Tet^R Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1lac Hte [F' pRPab $LACI^qZ$ Δm15 Tn10 (Tet^r) Amy Cam^r].	Agilent technologies
H37Rv (Wild type)	*Mtb* laboratory reference strain	Camus *et al*., 2002
Rv1268c Knock down	Derivative of H37Rv that carries the PURv1268 plasmid. Kan^R	This study

Tet^R : Tetracycline resistance, Kan^R: Kanamycin resistance

Table 2.2 Vector used in the study

Vector	Genotype	Reference
PLJR965	Crispr plasmid, with an inactive cas9 enzyme (dcas9) Controlled by an anhydrotetracycline (ATc)-inducible promoter. Kan^R	Rock *et al*., 2017

PURv1268	Derivative of PLJR965, carrying annealed oligos	This study

2.1.4. Bacterial growth conditions

2.1.4.1 Growth of *Mtb* strain

Mtb strains were grown in Middlebrook 7H9 liquid media supplemented with OADC, 0.5% glycerol, and 0.05% Tween 80 at 37 °C. Growth in solid media was conducted with 7H11 media supplemented with 0.5% glycerol, 79 g/L OADC enrichment and 25 mg/ml Kanamycin antibiotic was used.

2.1.4.2 Growth of *E. coli* XL10-Gold competent cells

E. coli XL-10-Gold was grown in Nutrient agar supplemented with 50 mg/ml Ampicillin at 37°C.

2.1.5 Gene Knock down

Gene silencing was achieved by using the clustered regularly interspaced short palindromic repeats (CRISPR) system. This system edits genes and is comprised of two major components, a guide RNA and CRISPR-associated protein 9 (Cas9) (Hsu *et al*., 2014). This genome editing system uses the Cas9 protein that is catalytically inactivated obtained from an organism known as from *Streptococcus thermophilus* ($dCas9_{sth1}$) (Rock *et al*., 2017; Qi *et al*., 2013). For this process, a 20 nucleotide bases sth1 single guide RNA (sth1 sgRNA) that is targeting either the promoter or the

open reading frame (Rock *et al*., 2017) and complementary to the non-template strand is designed to direct the sth1 dCas9 to the gene of interest (Rock *et al*., 2017; McNail and Cook, 2019).

A protospacer adjacent motif (PAM) that is appropriately placed identifies and binds the Cas9-sgRNA complex to the target site and results in the repression of transcription and, ultimately knocking down of the gene (Wright *et al*., 2016). The stronger the PAM sequence, the more the PAM will work efficiently for knocking down the gene (Rock *et al.*, 2017) and the strongest PAM is determined by the PAM sequence that is most similar to the consensus sequence 5' NNAGAAW-3' (Rock *et al*., 2017). The expression of the Cas9-sgRNA complex is controlled by an anhydrotetracycline (ATc)- inducible promoter that controls the gene knock down induction (Qi *et al*., 2013).

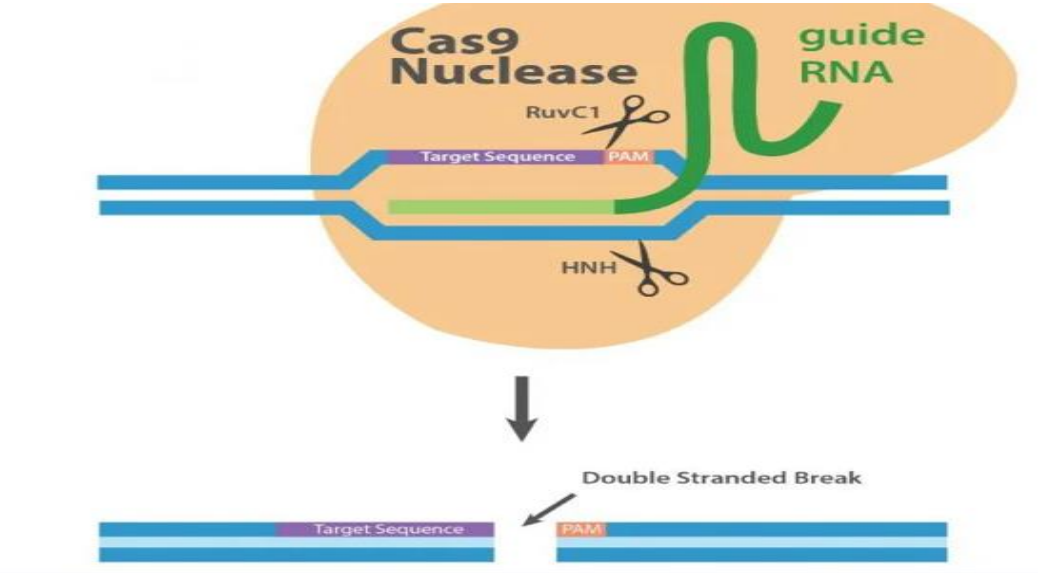


Figure 2.2. A diagram showing how the CRISPR technique works to knock down a gene. The Cas9 nuclease unwinds the double-stranded DNA close to the PAM sequence recognised by the sgRNA. The double-stranded break is then repaired by a non-homologous end joining pathway, which results in disruption in the open reading frame of the gene of interest (Nelson *et al*.,2019).

2.1.5.1 Plasmid extraction

An overnight culture of PLJR965 plasmid was done by adding 1 vial of PLJR965 freezer stock into 50 ml nutrient broth supplemented with 50 mg/ml kanamycin and incubated at 37°C with shaking (Polychem supplies SA,). PLJR965 plasmid was extracted using GeneJet plasmid Midiprep kit (Thermo Scientific, SA) as per the manufacturer's instructions. Briefly, the cells were harvested by centrifugation (Eppendorf Centrifuge 5415D) for 10 min at 5,000 ×g. The supernatant was then discarded, and the resulting pellet was resuspended in 2 ml of manufacture's resuspension solution. A volume of 4 ml of solution 2 was added and mixed, then incubated at room temperature for 5 minutes. Then 3 ml of solution 3 was added and mixed, followed by incubating on ice for 10 minutes. To pellet the cell debris and chromosomal DNA, centrifugation at 5000 ×g was done. The supernatant was then transferred to a 15 ml tube and 3 ml of 96% of ethanol was added and mixed by inverting the tube. Approximately 5.5. ml of the sample was transferred to the column pre-assembled with a collection tube.

Careful consideration was shown to prevent overfilling the column. The column was then centrifuged for 3 minutes at 2,000 ×g in a multifuge S-R (Thermo Scientific, South Africa (SA)). The supernatant was discarded, and the column was placed back into the same collection tube. To process any lysate that might have remained through the purification column, the previous step was repeated. Thereafter, 4 ml of wash solution I diluted with isopropanol was added to the purification column and centrifuged at 3000 ×g. The supernatant was discarded, and the column was placed back into the same collection tube. A volume of 4ml of wash solution II diluted with ethanol was added to the purification 6 column and then centrifuged for 2 min at 3000 ×g. The flow-through was discarded and the column was placed back into the same collection tube. The

previous step was repeated. Thereafter, the collection tube was centrifuged at 3000 ×g for 5 min, and then the collection tube with the flow-through was discarded. The column was then transferred into a fresh 15ml collection tube provided, then 0.35 ml of the elution buffer was added to the centre of the purification column membrane and incubated at room temperature for 2 min, followed by centrifugation for 5 min at 3000 ×g to elute plasmid DNA. The DNA yield was quantified by Nanodrop 2000 (Thermo Scientific, SA).

2.1.5.2 DNA Restriction

For restriction reactions, enzymes used were obtained from Thermo Scientific SA. A 20 µl reactions were set up following the manufacturer's instructions. Briefly, in a 1 ml Eppendorf tube, 10.5 µl distilled water, 2 µl restriction buffer, 2 µl BsmBI enzyme (10 U/µL), 1 µl DTT and 1 µg/µl plasmid were added and then gently mixed by pipetting up and down, followed by incubation at 37°C for 1 hour. After incubation, the reactions were heat-killed at 80°C using a heating block (FMH electronics, SA) for 10 minutes.

2.1.5.3 Agarose gel electrophoresis

The extracted and restricted PLJR965 plasmid DNA was run by agarose electrophoresis on a 0.8 % agarose gels in 1 × Sodium borate to separate molecular weight DNA. Electrophoresis was conducted in electrophoresis tanks (Bio-Rad laboratories, SA) containing 1 × Sodium borate buffer at 100 V. A 1 kb molecular weight marker (Thermo Scientific, SA) was used to determine the molecular weight of the plasmid DNA. Bands were visualised in the Syngene G:Box (Vacutec, SA) imaging system using Genesys V1.8.5.0. software.

2.1.5.4 Annealing Oligonucleotides

The Rv1268c forward and reverse primers were designed using clone Manager 9 Professional edition (Black Mesa laboratories Hack division). For the maximal gene knock down, the strongest PAM sequence was identified, and 23 nucleotides were extracted immediately 5' to the PAM sequence. To the primers selected, 5'-GGGA-3' sequence for the forward primer and 5'-AAAC-3' sequence for the reverse primer were added to create overhangs, which subsequently allow the oligonucleotides (oligos) to be incorporated into the PLJR965 plasmid after it has been digested with BsmBI during cloning. The primers have a GC content of 56% with 61.3% melting temperature. To anneal the oligos, a total of 50 µl volume reaction was set up containing 4 µl of both the forward oligonucleotide and the reverse oligonucleotide shown in Table 2.3 below, and 42 µl of annealing buffer made of 50 mM Tris pH 7.5, 50 mM NaCl and 1 mM EDTA. The reaction mixture was mixed by pipetting. This was followed by annealing in a T100^{TM} thermal cycler (Bio-Rad, SA) with the following programme 95°C for 2:00 and cool down for 0.1°C/sec to 25°C.

Table 2.3 Primers used in this study to anneal forward and reverse primers of RV1268c protein

Gene	Region	Primers
Rv1268c	Forward primer	GGGAGTTCCGCTATGCGGAGTGGATGT
	Reverse primer	AAACACATCCACTCCGCATAGCGGAAC

2.1.5.5 DNA ligation reactions

Ligation reactions were conducted with the use of T4 DNA ligase (New England BioLabs, England), following the manufacturer's instructions. Briefly, a total volume of 20 µl reactions contained 2 µl 10× T4 DNA ligase buffer, 1 µl vector DNA, 0.5 µl insert DNA, up to 20 µl distilled water and 1 µl T4 DNA Ligase. Different molar ratios of vector: insert DNA (1:0, 1:1, 1:2 and 1:3) were set up for the ligation reactions. The reactions were then incubated in a T100^{TM} thermocycler (BIO-RAD, SA) at 22°C for 2 hours. Figure 2.2 below shows the map that shows how the annealed oligos are ligated to PLJR965.

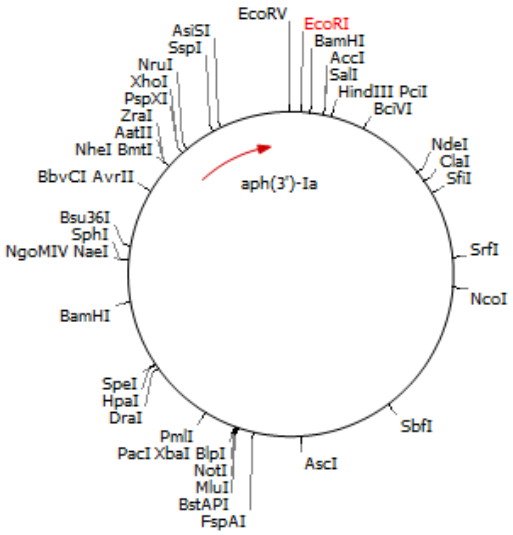


Figure 2.3. Plasmid map of PURv1268 illustrating the annealed oligos cloned into PLJR965 backbone

2.1.6. Transformation

2.1.6.1 Preparation of ultracompetent cells

A single colony of *E.coli* XL10-Gold cells grown on nutrient agar plates was inoculated into 10 ml of nutrient broth and then incubated at 37°C overnight. A volume of 1 ml overnight culture was added into 50 ml nutrient broth and incubated at 37°C for 2-3 hours. The culture was transferred into a 50 ml falcon tube and allowed to stay on ice for 10 minutes. The culture was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml of 100 mM ice-cold calcium chloride and transferred into a clean Eppendorf tube. The resuspended pellet was centrifuged for 30 seconds, and the supernatant was discarded. The pellet was resuspended in 1 ml of 100 mM calcium chloride, followed by incubation on ice for 2 hours.

2.1.6.2 Transformation into *E.coli* XL10-Gold cells

Transformation was carried out following the manufactures instructions (Agilent Technologies, United States). Two 14 ml BD Falcon polypropylene round-bottom tubes were pre-chilled on ice, and NZY^+ broth was preheated to 42°C. The ultracompetent cells (2.6.1) were gently mixed, and 100 µl was aliquoted into each of the two pre-chilled tubes. A volume of 4 µl of Beta-mercaptoethanol (Agilent Technologies, United States) was added to each aliquot of cells. The tubes were gently swirled and then incubated on ice for 10 minutes, swirling gently every 2 minutes. A volume of 2 µl of ligation mixture was added to one aliquot of cells. pUC19, a double -stranded circular plasmid cloning vector that is used in *E.coli* (Vieira and Joachim, 1982) was used as a control DNA. pUC19 was diluted 1:10 with sterile distilled water, and then 1 µl of pUC19 vector DNA (Thermofisher Scientific, SA) was added to the other aliquot of cells. The

tubes were gently swirled and incubated on ice for 30 minutes. The tubes were heat-pulsed in a 42°C water bath for 30 seconds following incubation on ice for 2 minutes. A volume of 0.9 ml of pre-heated NZY^+ broth was added, and then the tubes were incubated at 37°C for 1 hour with shaking at 225-250 rpm. A volume of 200 µl of the transformation mixture was plated on nutrient agar plates supplemented with kanamycin. For the pUC19 control transformation, 5 µl of the transformation reaction was plated on nutrient agar plates supplemented with ampicillin. The plates were incubated at 37°C overnight

After the incubation, colonies were randomly picked and screened by restricting with BamHI (Thermo Fisher Scientific, SA) as the primers contained a restriction site for BamHI and then ran on 0.8% agarose gel, expecting two molecular weight sizes of, 6070 bps and 2569 bps. The one positive clone was grown in 50ml kanamycin-containing broth overnight, followed by maxi plasmid extraction and then ran on agarose gel. The extracted plasmids were stored at -20°C for future use.

2.1.7 Electroporation into *Mtb*

A single colony of *Mtb* plated on agar plates was used to grow a 5 ml culture overnight at 37°C. This culture was inoculated into 50 ml 7H9 media and grown to an absorbance (OD600nm) of 1, measured by a spectrophotometer. The cells were harvested by centrifuging at 3500 × g for 10 min at 4 °C, and the supernatant was discarded. Thereafter, the pellet was washed 4 times by resuspending in successive steps of 45 µl, 20 µl, 10 µl and 5 µl of ice-cold 10 % glycerol, then centrifuged at 3500 × g for 10 min at 4 °C. The cell pellet was then resuspended in 2 ml ice-cold

10 % glycerol, and the competent cells were immediately used for electroporation. Different concentrations of vector DNA (1 µg, 3 µg and 5 µg) were added to a 400 µl aliquot of *Mtb* competent cells and transferred to a 0.2 cm electroporation cuvette, followed by electroporation using the following conditions: 2.5 kV, 25 µF and 1000 Ω using an Electro Cell Manipulator (ECM 630) (Fisher Scientific, SA). A volume of 800 µl of 7H9 media was added to the cells immediately after pulsing. The cultures were then incubated at 37°C overnight. The overnight cultures were plated on Middlebrook 7H10 media containing kanamycin. The plates were left to dry for 2 hours and incubated at 37 °C for 3 – 4 weeks.

2.1.8. Small-scale DNA extraction for PCR

A single colony was picked from a grown culture plate of electroporation products and added to 40 µl of water. The 40 µl volume was separated into two 20 µl volumes, 1 ml of 7H9 broth supplemented with kanamycin antibiotic was added into one 20 µl and was incubated at 37°C to be later used to make stock solutions. The other 20 µl was heat activated at 95°C for 5 minutes. The culture was then washed with 40 µl of phenol-chloroform and centrifuged at 400 × g for 5 minutes. The top layer was transferred into the new Eppendorf tube and then used as DNA.

2.1.9 PCR screening

The extracted DNA was used for PCR screening. Colonies were screened if they contained the knock down plasmid using a specific Rv1268c forward primer and a universal primer listed in Table 2.4. A 20 µl reaction containing 2,5 µl of Taq buffer, 2,5 µl dNTPs, 1,25 µl forward primer, 1.25 µl universal primer, 2 µl DNA (50ng), 0,25 µl Taq polymerase and 15,25 µl of distilled water,

was set up and placed in a T100^{TM} thermocycler (Bio-Rad, SA) with the following conditions: initial denaturation at 95˚C for 3 minutes, 95˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 1 minute, 72˚C for 2 minutes and hold at 16˚C for 10 minutes. The expected molecular weight was 504 bps as a confirmation that the knock down was incorporated in *Mtb*. The amplicons were then electrophoresed on a 2% agarose gel electrophoresis.

Table 2.4 Primers used for screening of Knock down plasmid

Gene	**Region**	**Primers**	**Amplicon properties**
PURv1268	Specific Forward primer	GGGAGTTCCGCTATGCGGAGTGG ATGT	504 bp amplicon; specific forward primer binds on the knock down from base pair 174 to 196 and reverse primer binds on PLJR965 base pair from 654 to 673
	Universal reverse	GGAGAAAGGCGGACAGGTAT	

2.2. Phenotypic characterisation of Rv1268c

2.2.1 Growth rate analysis

The bacterial growth of the *Mtb* wild type, knock down, and a knock down induced with 100 ng of anhydrotetracycline (aTc) was analysed by growth curve experiment. aTc was added to inhibit the Rv1268c gene expression by binding to the Tet promoter to destruct the polymerase from transcribing the protein. 10 ml culture volumes for each strain were set up using a single colony plated on nutrient agar and grown at 37 ˚C until they reached the stationary phase (OD600nm ≈ 2). The cultures were then diluted to a final absorbance (OD600nm) of 0.05 in 50 ml 7H9 broth with Tween 80, OADC, glycerol and kanamycin antibiotic for the mutant and no antibiotic for the wild-type strain. The cultures were incubated at 37 ˚C. The assay was done in triplicates to verify the results. The *Mtb* i*n vitro* growth was determined by measuring absorbance (OD600nm) using spectrophotometer every 24 hours for 14 days.

2.2.2 Ethidium bromide diffusion (EtBr) assay

Fluorometric Ethidium bromide, which is recognized as a substrate for many efflux pump systems, was utilised for the efflux assay to test if Rv1268c can facilitate the transport of molecules coupled to ATP hydrolysis. EtBr assay is based on the principle that when fluorescent substances are utilised at a sub-lethal level, enter the cell through passive diffusion, and can be taken out through active efflux (Sharples and Brown, 1976). Cultures (20 ml) of the wild type, knock down and a knock down induced with 100ng of aTc were grown in 7H9 liquid media that contained kanamycin for mutant strains, to an absorbance (OD600nm) of 0.8. The cells were collected by

centrifugation at 3900 × g for 10 min, following washing twice with 1 ml PBS and resuspended in 1 ml PBS that contained 0.4% glucose. The cells were then adjusted to an OD600nm of 0.4. A volume of 95 µl of the bacterial sample adjusted was added into each well of a 96 well plate, and then EtBr at a final concentration of 8 µg/ml was added. The experiment was done in triplicates to validate the results. Fluorescence was measured with an excitation of 530 nm and emission wavelength of 585 nm every 60 seconds for 10 min at 37 °C.

2.2.3 Antibiotic susceptibility assay

The Minimum Inhibitory Concentration (MIC) of antibiotics for the *Mtb* wild type, knock down and a knock down induced with 100ng of aTc was determined using a microtiter assay. A number of 10 different concentrations were tested for each antibiotic. The antibiotics that were used were ofloxacin (25,6 µg, 12.8 µg, 6.4 µg, 3.2 µg, 1.6 µg, 0.8 µg, 0.4 µg, 0.2 µg, 0.1 µg and 0.05 µg), erythromycin (160 µg, 80 µg, 40 µg, 20 µg, 10 µg, 5 µg, 2.5 µg, 1.25 µg, 0.626 µg and 0.3125 µg), meropenem (128 µg, 64 µg, 32 µg, 16 µg, 8 µg, 4 µg, 2 µg, 1 µg, 0.5 µg, and 0.25 µg) , colistin (32 µg, 16 µg, 8 µg, 4 µg, 2 µg, 1 µg, 0.5 µg, 0.25 µg, 0.125 µg and 0.0625) and ciprofloxacin (25,6 µg, 12.8 µg, 6.4 µg, 3.2 µg, 1.6 µg, 0.8 µg, 0.4 µg, 0.2 µg, 0.1 µg and 0.05 µg). Briefly, bacterial strains were grown in 7H9 broth at 37°C to an absorbance (OD600nm) of 0.3.

A 96 well plates were set-up. Antibiotic stocks that contained 4 × the initial concentration that was added for the first wells in the plate were prepared, and then 100 µl of each stock was inoculated into the first wells. Afterwards, 50 µl of 7H9 broth was inoculated into wells 2 to 12. A 50 µl aliquot of the antibiotic stock from well 1 was inoculated into well 2, mixed and 50 µl was

removed from well 2 and inoculated into well 3. This was done until well 12 to create 2-fold serial dilutions of antibiotics. A volume of 50 µl aliquot was removed from the last well and discarded. Bacterial cultures were then diluted 10 000-fold and 50 µl aliquot was added to each well. Controls experiments involved the same treatments without antibiotics. The plates were sealed and incubated at 37˚C for 7 days, after which growth was examined to determine the MIC values.

2.2.4 Statistical analysis of the results

The two-sample t-test was used to compare the results of the wild type to the knock down. The significant difference was determined with Probability-value test using the average of the triplicate samples for the wild type and the knock down.

CHAPTER THREE

RESULTS

3.1 Bioinformatics analysis of Rv1268c

The Rv1268c gene encodes a 232 amino acid protein with a molecular weight 23973 kilo Daltons (Song *et al*., 2008). Through mutagenesis analysis, Rv1268c was proven to be a non-essential gene for *in vitro* growth of H37Rv when grown in MtbYM-rich medium (Minato *et al*, 2019). Rv1268c has 2 domains, a Peptidase_C39_2 domain (IPRO39564) and a peptidase C39-like protein domain (IPRO39564), as shown in appendix 6.4D (**Figure D1**). These belong to the peptidase family and are found in a wide range of ABC transporters. This suggests that Rv1268c might be an ABC transporter. InterPro functional analysis revealed that, Rv1268c is predicted to have a region of a membrane-bound protein that is predicted to be outside of the membrane, in the external cellular region and contains signal peptides which suggest that Rv1268c is a secreted protein (**Appendix 6.4D, Figure D1**).

The protein-protein analysis done using String software showed that the Rv1268c protein is predicted to be co-expressed and co-exists with Rv1269c, which is a conserved probable exported protein. Rv1268c is also predicted to co-occur with Rv0713, a probable conserved transmembrane protein as shown in the appendix 6.4D (**Figure D3**). Analysis with Phyre2 identified six structural homologues of Rv1268c, namely, GBAA-0574, LahT150, SMU-286, Cthe-0534, Alr0975, Pcal3 (**Figure 3.1**). Out of these 6 structural homologues, GBAA_0574 is the closest match with 99.6% of confidence. The confidence percentage depicts the probability (from 0 to 100) that the match between the Rv1268c sequence and GBAA_0574 are intimately related.

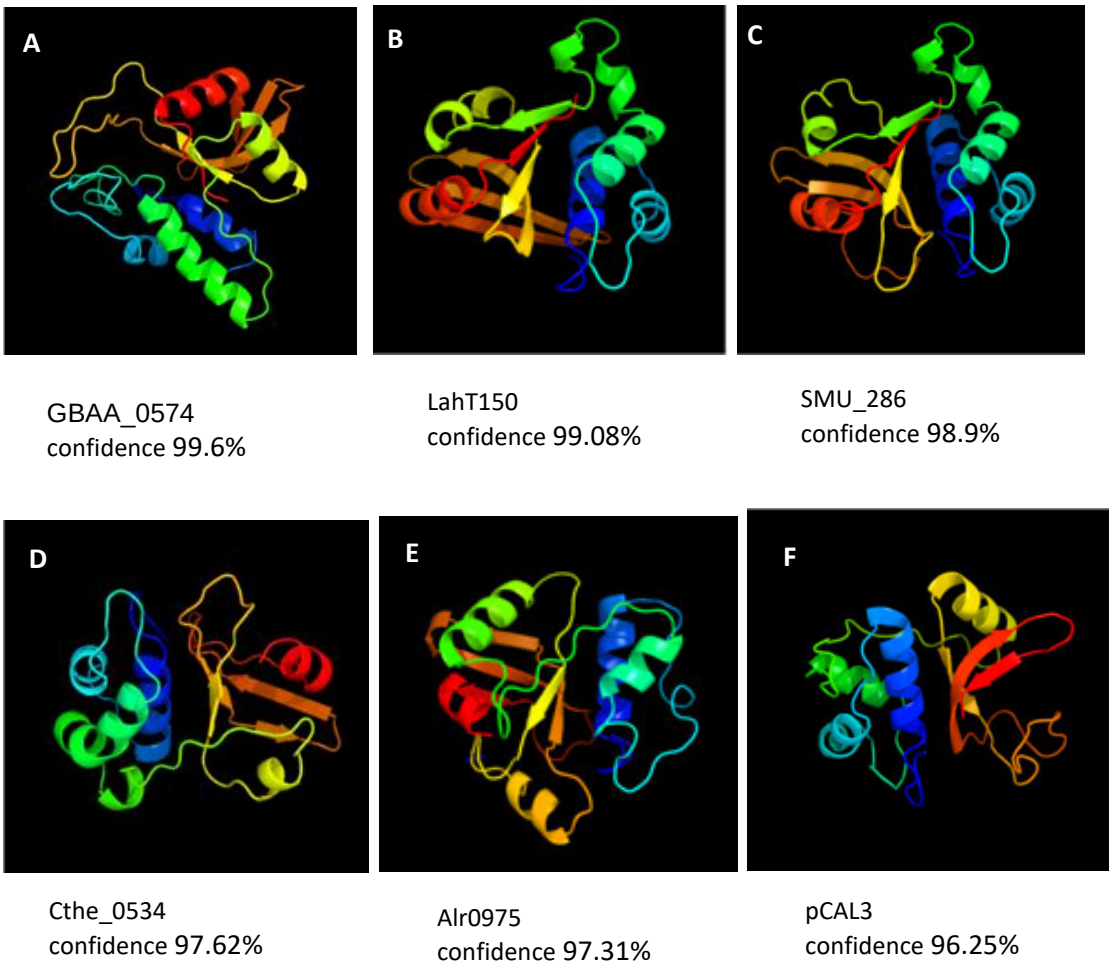


Figure 3.1: Schematic representation of functional and structural homologs of Rv1268c identified by Phyre2. **A)** GBAA_0574 from *Bacillus anthracis* with confidence 99.6% **(B)**. LahT150 from *Lachnospiraceae bacterium C6A11* and *Prochlorococcus marinus str*. *MIT 9313* with confidence 99.08% **(C).** SMU_286 from *Streptococcus mutans* with confidence 98.9% **(D)** Cthe_0534 from *Acetivibrio thermocellus ATCC 27405* with confidence 97.62% **(E)** Alr0975 from *Nostoc sp. PCC 7120=FACHB-48* with confidence 97.31% **(F)** pCAL3 from *Tenebrio molitor* with confidence 96.25%

3.2. Construction of knock down mutant in Rv1268 gene

Rv1268c was knocked down to study its physiological role in the growth and survival of *Mtb*. The *Mtb* mutant with the Rv1268c gene knocked down was created by using a CRISPR system.

3.2.1 Construction of vector for knock down of Rv1268c

PLJR965 was extracted and then electrophoresed to confirm the success of the extraction. The **Figure 3.2** below shows the successful extraction of the circular PLJR965 DNA, as a DNA band of the expected size (8631 bps) was observed.

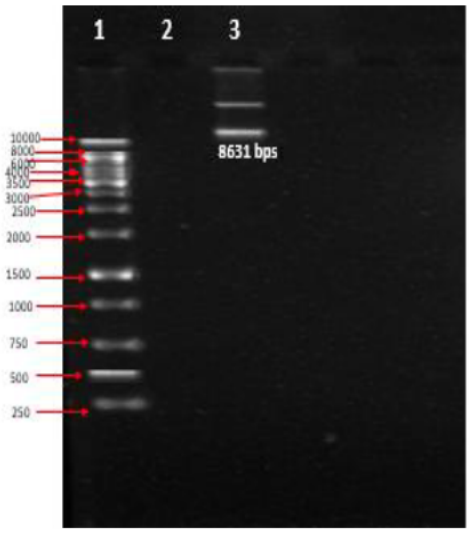


Figure 3.2. Extraction of PLJR965. Agarose gel electrophoresis showing a circular PLJR965 DNA that results from the extraction of plasmid PLJ965. The gel shows **[Lane 1]**: 1Kb DNA Ladder, **[Lane 3]**: extracted PLJR965.

The PLJR965 plasmid was restricted with BsmBI, and the results confirms that PLJRR965 was linearized, as the fragment size corresponds to the expected size of 8,606 bps (**Figure 3.3**). The restricted plasmid was then ligated with Rv1268c annealed oligos and subsequently transformed into XL-10 Gold cells and incubated overnight at 37°C as described in **section 2.6.2**.

A

Enzymes	**Expected size (bps)**
Uncut PLJ965	8631
BsmBI	8606

B

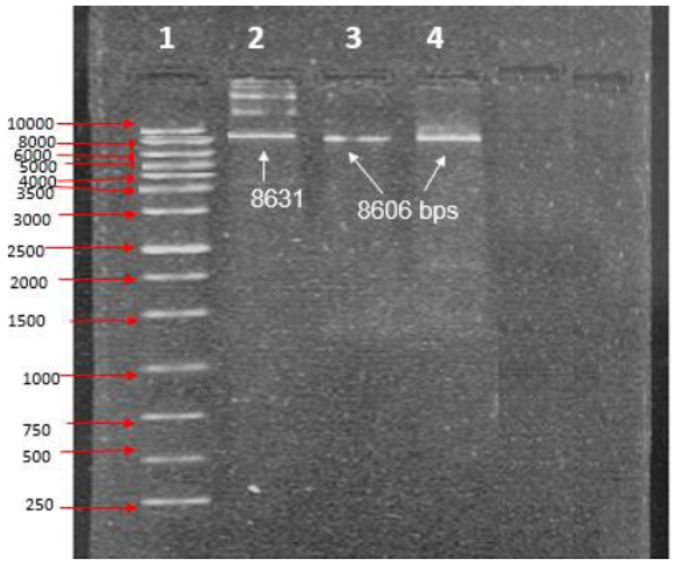


Figure 3.3 Restriction profile of PLJR965. **(A)** Table showing the used restriction enzyme and expected fragment band size. **(B)** Agarose gel electrophoresis displaying fragment size of the restricted PLJR965 plasmid with BsmBI, **[Lane** 1]: 1kb DNA Ladder, **[Lane 2]**: Uncut PLJR965, **[Lane 3 and 4]**: PLJR965 cut with BsmBI.

Colonies that potentially contained the desired plasmid were screened by restriction profiling. The PURv1268 plasmid was extracted with an expectation of 8,639 bps and then it was restricted with BamHI, expecting two band sizes of 6,070bps and 2,569 bps (**Figure 3.4**). The restricted plasmid was run alongside PLJR965 cut with BamHI and EcoRI because they separate at the same molecular size.

The PURv1268 plasmid was successfully extracted from only one colony as the fragment size of 8,639 bps corresponds to the knock down plasmid map shown in **Figure 2.3**. The one clone selected was confirmed by restriction profiling (**Figure 3.4**) because the expected fragments 6,070 bps and 2,569 bps were obtained. There were no missing or extra bands displayed which further confirms the integration of Rv1268c into PLJR965 plasmid.

A

Enzymes	**Expected sizes (bps)**
Uncut PLJR965	8639
Uncut PURv1268	8639
PURv1268 cut with BamHI	6070 and 2569
PLJR965 cut with BamHI and EcoRI	6138 and 2493

B

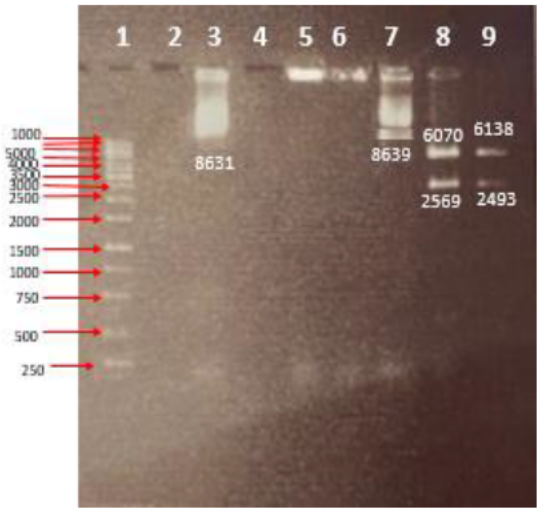


Figure 3.4 Restriction profile of the knock down plasmid, PURv1268. **(A)** Table showing the used restriction enzymes and expected band sizes **(B)**. Agarose gel electrophoresis showing fragment sizes from restrictiona of plasmid PURv1268, **[Lane 1]**: 1kb DNA Ladder, **[Lane 3]**: Uncut PLJR965, **[Lane 7]**: uncut PURv1268 **[Lane 8]** PURv1268 cut with BamHI and **[Lane 9]**: PLJ965 cut with BamHI.and EcoRI

3.3 PURv1268 integration into *Mtb* H37Rv genomic DNA

PURv1268 was electroporated into *Mtb* H37Rv. After 2-4 weeks of incubation, the colony that potentially carried the plasmid insert was selected, and plasmid DNA was subsequently extracted from the clone and screened by PCR using specific a primer and a universal primer as described in **section 2.9**. PCR screening confirmed the PURv1268 integration into *Mtb*. The expected fragment band size of 504 bp was obtained **(Figure 3.5)**, The mutant was then used for phenotypic characterisation.

A

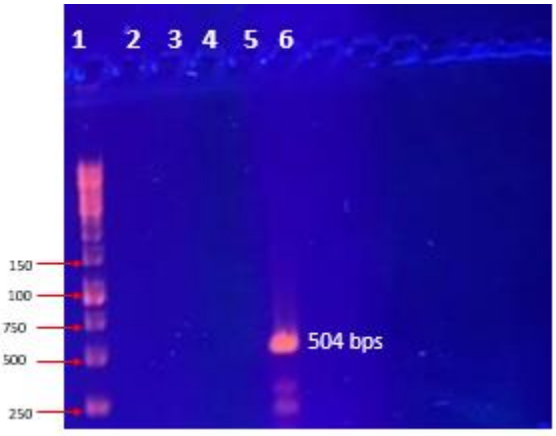


Figure 3.5 Screening of the PURv1268 electroporated product **(A)** agarose gel electrophoresis displaying fragments that result from the integration of PURv1268 in *Mtb* genome, **[Lane 1]**: 1kb DNA Ladder, [**Lane 9]**: PCR product

3.4 The functional characterization of RV1268c, to elucidate its role on the survival of *Mtb*

Different assays to study the effect impacted by knocking down Rv1268c on the growth and survival of *Mtb* were conducted. The three assays conducted were growth analysis, Minimum inhibitory concentrations of selected antibiotics, and ethidium bromide assay. The assays were conducted on the wild type, knock down, and the knock down with 100ng of aTc to control the transcriptional activity.

3.4.1 RV1268c has no effective role in the growth of *Mtb*

To assess the effect of the knocked down Rv1268c on the *in vitro* growth of *Mtb*, growth assay was set up as mentioned in **section 3.1.1**. The results were represented using a line graph, as shown in **Figure 3.6** below.

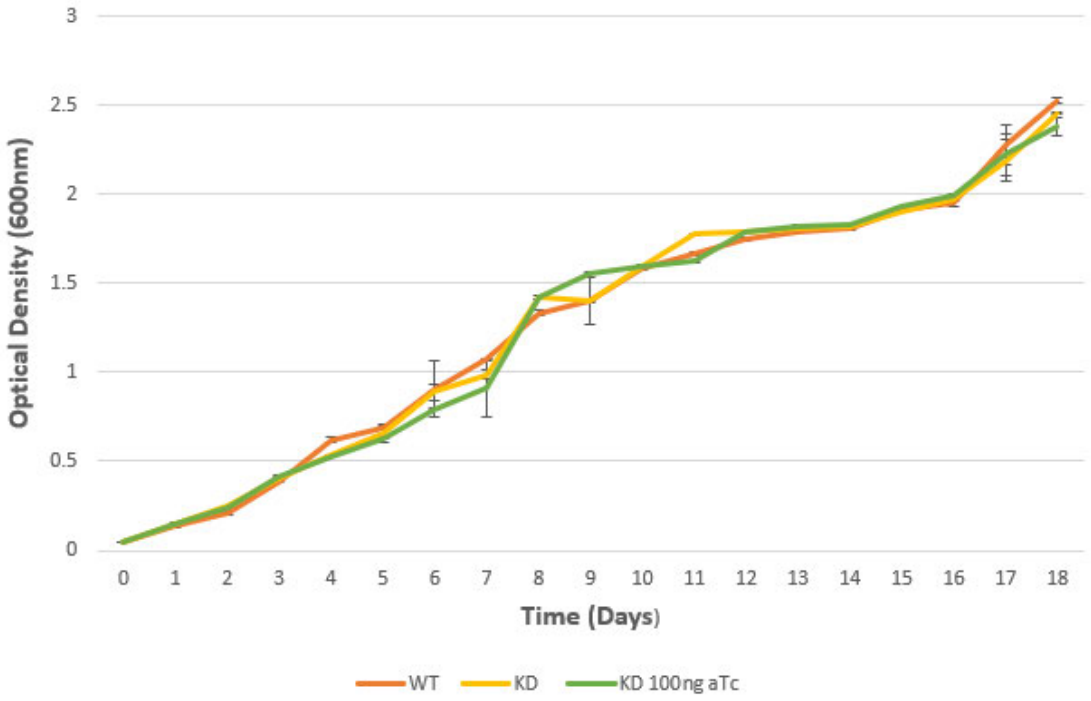


Figure 3.6. Growth analysis of the *Mtb* wild type, Rv1268c knock down, and Rv1268c knock down with aTc. Displayed in the graph is the increase in OD600nm over a period of 18 days. Each point corresponds to the average of the determinations.

All three strains displayed comparable survival. The logarithmic growth phase lasted 7 days for the wild type, 6 days for the knock down and 6 days for the knock down with aTc **(Figure 3.6)**. Even though the wild type displayed a slightly higher cell yield in day 4, the density of all the three strains rose to similar levels irrespective of the presence of aTc in the knock down. This means that all three strains grow at a similar rate. A t test was done to compare the different time points and there was no significant difference between the growth of the WT and mutants with and without aTc.

3.4.2 Rv1268c does not play a role in the transportation of molecules coupled to ATP hydrolysis.

An EtBr assay was conducted to study if Rv1268c plays a role on the extrusion of molecules. Results were represented in a line graph as shown in **Figure 3.7** below, and the points of significance are represented by the symbol **

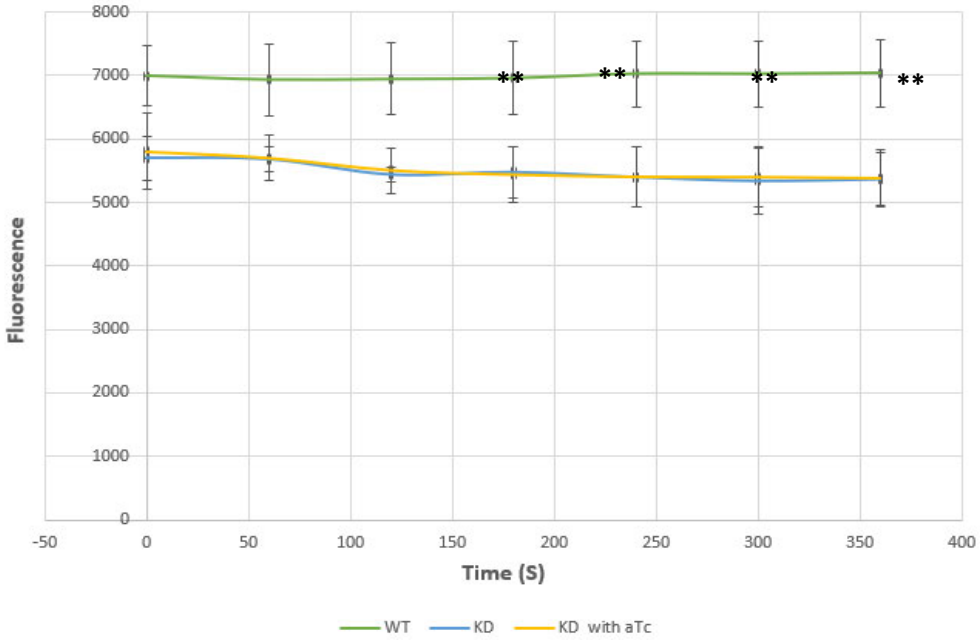


Figure 3.7 EtBr diffusion in the Rv1268c knock down, wild type (WT), and the knock down with 100ng of aTc over a period of 360 s. The results were considered significant (**) at $p < 0.05$.

Much greater fluorescence was observed in the wild type compared to the knock down and the knock down with aTc **(Figure 3.7)**. The two mutant strains displayed a similar pattern of EtBr extrusion. This was further confirmed by the t test (results not shown), with a p value of 0.033. A t test showed that there was a difference at 180 seconds with p=0.03, at 240s with p=0.004, at 300s with p=0.004, until 360 seconds with p=0.003. This suggests that Rv1268c does not play

role in the transport of molecules because the higher fluorescence in the wild type means that the EtBr was not extruded, instead, it accumulated inside the cell.

3.4.3 EFFECT OF Rv1268c ON DRUG SUSCEPTIBILITY

A minimum inhibitory concentration (MIC) assay was performed to determine the drug susceptibility of the wild type, knock down, and the knock down induced with 100ng aTc. The MIC assay was set up as described in **section 3.3** using the following drugs: ofloxacin, erythromycin, meropenem, coliston, and ciprofloxacin. The MICs were then plotted on the bar graph in **Figure 3.8** below.

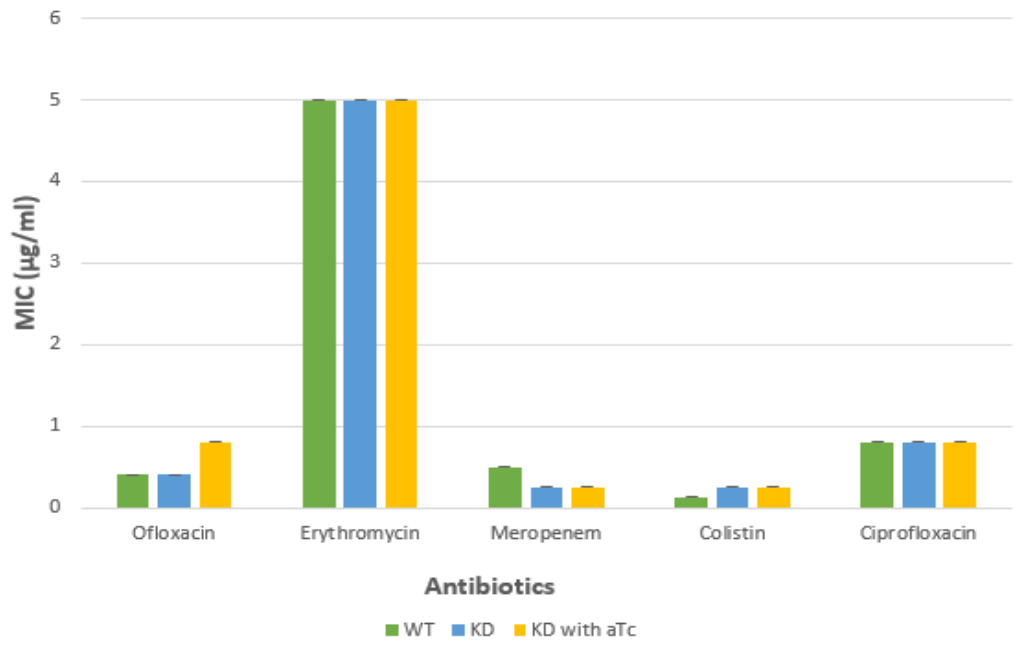


Figure 3.8. Drug susceptibility of the wild type, knock down, and knock down with aTc strains to several drugs. Displayed in the graph are the MICs of the wild type (green bars), knock down (blue bars) and the knock down with aTc (mustard bars) to various antibiotics. Error bars were determined by using the standard deviation of the triplicates of cultures and plotted on the graph as Y-axis error bars.

Neither the wild type, knock down nor knock down with aTc displayed any alternation in the sensitivity to erythromycin and ciprofloxacin. The knock down with aTc displayed a higher MIC for ofloxacin, and the wild type displayed lower MIC in colistin as compared to the mutant strains. The knock down and knock down with aTc displayed lower MIC to meropenem and as compared to the wild type (**Figure 3.8**), which could suggest that Rv1268c functions as a drug transporter. However, the statistical analysis (data not shown) showed that there was no significant difference as a p-value of 0.6299 > (0.05), was obtained. The growth of none of the strain was completely inhibited by all of the drugs tested.

CHAPTER FOUR

DISCUSSION

Tuberculosis (TB) is among the top 10 causes of death globally and was the leading cause of death until the emergence of the Covid-19 pandemic in 2020 (WHO, 2020). Numerous anti-Tb drugs have been discovered and were effective until the emergence of drug resistant *Mtb* strains (WHO, 2011). Resistance to different environmental stresses has been linked with the ability of *Mtb* to survive within their hosts (Manganelli *et al*.,2004). In the previous ten years, a number of proteins associated with drug resistance were discovered, including proteins in the ATP-binding cassette (ABC) transporter family (Gustot *et al*.,2010; Rodriguez-Garcia *et al*.,2006). ABC transporters play a vital role in the maintenance of cellular homeostasis of *Mtb* by translocating molecules in and outside of the cells (Braibant *et al*., 2000). They can also function as efflux pumps and can contribute to antibiotic resistance (Singh *et al*., 2020).

Since some of these proteins play a role in drug resistance, there has been an increase in applied research that aims to understand better their mechanism of molecule transportation (Loo *et al*.,2002). The antibiotic producer's ability to self-defence relies on their endogenous drug resistance proteins, hence these resistance proteins may serve as antibiotic production targets (Qiu *et al*.,2012; Malla *et al*.,2010). This study reports the characterisation of the biological function of the Rv1268c, which is hypothesised to be an ABC transporter (Song *et al*.,2008). Elucidating the function of this protein will help with the overarching objective of producing more effective anti-TB drugs to lessen the global burden of TB. Rv1268c gene expression was repressed by CRISPR, and phenotypic assays were carried out to assess the effect of the gene on *Mtb* growth and when the protein was silenced.

In silico studies showed that Rv1268c, a hypothetical protein, encodes a 232 amino acid protein with a molecular weight 2,3973 kiloDaltons (Song *et al*., 2008). Rv1268c has 2 domains, a Peptidase_C39_2 and peptidase C39-like protein domain. These function to cleave the double-glycine leader peptides from the precursors of different bacteriocins (non-lantibiotic) (Barrett and Rawlings, 2001). The closest structural homolog of Rv1268c, identified as GBAA_0574 from *Bacillus anthrasis*, also belongs to the putative C39-like peptidase family where this domain is found in a wide range of ABC transporters. Like Rv1268c, GBAA_0574 is also a hypothetical protein with an unknown function (Bonanno *et al*.,2008). Rv1268c has been predicted to have a region of a membrane-bound protein in the extracellular region (Song *et al*., 2008). External membrane-bound proteins function to transport molecules across the membrane and to or anchor the cell to the extracellular space (Robb and Campton, 2021). Further analysis was done to investigate the protein-protein interactions of Rv1268c, and it was found to coexist with Rv0713c and Rv1269c.

Rv0713c was identified in whole cell lysates of *Mtb* and predicted to be a conserved transmembrane protein with no defined function (de Souza *et al*.,2011). Rv1269c was also identified in culture filtrates of *Mtb* and is predicted to be a secreted protein, also with unknown function (Malen *et al*., 2007). Based on bioinformatics analysis of this study, Rv1268c could function as an ABC transporter. To support this *in vitro* study, phenotypic characterisation was conducted.

To validate the results obtained in the bioinformatics study, the growth mechanism of the knock down was compared to that of the wild type. In liquid culturing conditions, the knock down, wild type, and the knock down with aTc results showed no significant difference in growth across the two weeks for which this was evaluated. (Figure 4.6). To confirm these results, a statistical analysis to compare the significant difference was conducted using a t-test where the probability value (P-value) is > 0.05 indicating that there is no significant difference in growth between the wild type and the knock down mutant. Thus, knocking down Rv1268c has no effect on the ability of the *Mtb* to utilise and metabolize nutrients required for its *in vitro* growth. This corroborated previous studies by Minato *et al*, 2019, which showed that the Rv1268c mutant was proven to be a non-essential gene for *in vitro* growth of H37Rv when grown in MtbYM-rich medium.

Subsequently we studied if Rv1268c played any role in the translocation of molecules. Various assays to study transport processes are employed but fluorescence experiment using ethidium bromide as a substrate is the well-utilized method for assessing efflux activities (Greulich, 2004; Jernaes and Steen, 1994; Martins *et al*., 2006). The EtBr experiment was conducted to check if EtBr was going to be extruded outside the cell. Mycobacteria have active efflux pumps, which play a role in natural drug resistance and EtBr has been proven to be pumped out of mycobacterial cell walls (Danilchanka *et al*., 2008). EtBr is a fluorescent molecule that enters the cell by passive diffusion and can only be removed by an active efflux system (Blair and Piddock, 2016). Comparing the knock down and the knock down with aTc to the wild type, the results displayed a significant difference in the fluorescence measured (Figure 4.7). The wild type had higher

fluorescence as compared to the knock down and the knock down with aTc, where both mutants displayed comparable fluorescence levels. Since EtBr fluoresces when it is bound to DNA, its fluorescence is higher when it is inside the cell. Therefore, the wild type displayed higher fluorescence because EtBr accumulated within the cell, meaning it was not extruded outside the cell, unlike in the knock down and the knock down with aTc, they both had decreased fluorescence which possibly means that in the absence of the active Rv1268c, EtBr was extruded from the cell to the cytoplasm. This contradicts the hypothesis that Rv1268c is an ABC transporter, because in such an instance, since the Rv1268c expression was repressed, fluorescence would have been higher than the wild type. This is because the EtBr would have accumulated within the cell, because Rv1268c is not active, hence EtBr would have not been extruded. The findings of this study suggests that the Rv1268c protein inhibits the transport of EtBr, since in the presence of this protein EtBr is extruded and in its inactive form, EtBr is extruded.

Since Rv1268c has been proven in this study not to have an effect on the growth of *Mtb* and displayed not to play a role in the transport of EtBr, this protein was further characterised to study the susceptibility patterns of *Mtb* to various drugs. Although *Mtb* has numerous efflux pumps, only a few of them have been proven to modulate susceptibility to anti-TB agents (Poole, 2007). The function of efflux in modulating drug susceptibility depends on both the drug and the bacterium, hence efflux pumps have an essential effect on the survival of the bacteria (Piddock *et al*.,2006).

The antibiotics used in this study were chosen based on their clinical importance. Oflaxicin and ciprofloxacin are fluoroquinolones that inhibit the enzymes DNA gyrase which is essential for the

DNA replication (Guler and Erac, 2016). Fluoroquinolones are currently the most recent class of antibiotics that offer hope in the anti-TB drug discovery (Berning, 2001). WHO has advocated for ofloxacin in a case of MDR-*Mtb* if the resistance has been proven to both isoniazid and rifampicin (Walwaiker *et al*.,2003). Erythromycin is from a class of macrolides, which are suggested to be researched more because they are promising anti-TB drugs (Anne-Fleur *et al*., 2015). This is because of their ability to accumulate in the bacterial compartments and cells, they are immunomodulators and have a synergic effect with other anti-TB antibiotics (Anne-Fleur *et al*., 2015). Meropenem is carbapenem which has a bactericidal effect by inhibiting the peptidoglycan cross-linking that is associated with the cell wall syntheses and results in cell death (Cho *et al*., 2018).

The wild type, knock down and the knock down with aTc displayed no differences on the MICs to erythromycin and ciprofloxacin, while the wild type showed a reduction in the amount of antibiotic that is needed for growth inhibition to meropenem and colistin as compared to the knock down and knock down with aTc. The statistical analysis showed that there was no significant difference as a p-value of 0.6299 was obtained, therefore the results of drug susceptibility profiling suggest that Rv1268c does not play a role in drug resistance. This is because if Rv168c played a role in drug resistance, growth would have been observed in the wild type for the tested antibiotics irrespective of the concentrations used. Also, a higher MIC would have been observed which would be needed to inhibit *Mtb* growth.

CONCLUSION

In conclusion, our study showed that *Mtb* Rv1268c is not an ABC transporter. This is because the knocking down of the gene did not show any impact in the *in vitro* growth of *Mtb*, in the transportation of EtBr and did not show any complete resistance in all the drugs tested.

To further validate the hypothesis that RV1268c is an ABC transporter, further studies of Rv1268c are advised to further characterise the protein. This can be done by employing other different techniques such as gene knock out and more physical characterization techniques such as ATPase Activity determination. The findings of those future studied would be a comparison to the results of this study which showed that Rv1268c is not an ABC transporter.

6. Appendices

6.1 Appendix A: Culture media

Nutrient Agar

15.5g Nutrient agar, 500ml sdH_20, 0.5ml of 25 mg/ml Kanamycin (where needed)

Nutrient Broth

15g Nutrient broth, 1L sdH_20, 1ml of 25 mg/ml Kanamycin (where needed)

NZY^+ Broth

10g NZ amine, 5g yeast extract, 5g Nacl, 1Ltr sdH_20, adjusted to pH 7.5 then autoclaved, filter-sterilized 12.5ml 1M $MgCl_2$, 12.5ml 1M $MgSO_4$, 20ml 20% glucose

Middlebrook 7H9

2.35g Difco Middlebrook 7H9 powder, 2ml glycerol, 450ml sdH_20, 1ml Tween80

Middlebrook 7H10

9,5g Middlebrook 7H10 powder, 2.5ml glycerol, 450ml sdH_20, 50ml of 79 g/L OADC, 0.5ml of 25 mg/ml Kanamycin

Tween80 (25%)

10ml Tween 80, 40ml sdH_20 then sterilized by filtration

6.2 Appendix B: Solutions

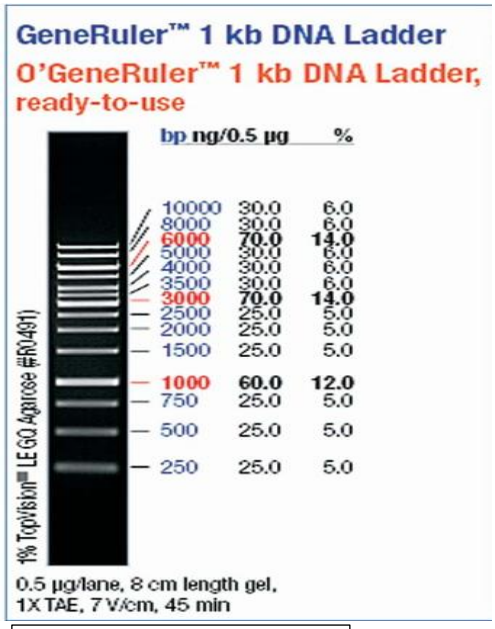
DNA manipulation solutions

Annealing buffer 50mM Tris (pH 7), 50mM NaCl, 1mM EDTA

Sodium borate (20X) 8g NaOH, 48g boric acid, 900ml sdH_20, pH 8, 1000ml final volume sdH_20

Sodium borate (1X) 50ml 20X sodium borate, 950ml sdH_20

6.3 Appendix C: Molecular weight marker



1KB DNA Molecular weight marker

Figure C1. 1kb DNA molecular weight marker

6.4 Appendix D: Bioinformatic analysis

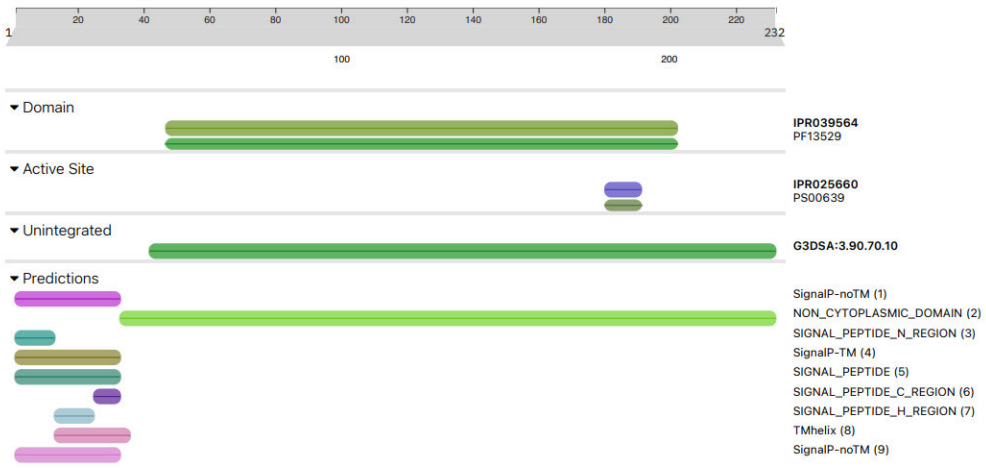


Figure D1: InterPro analysis of the *Mtb* Rv1268c gene

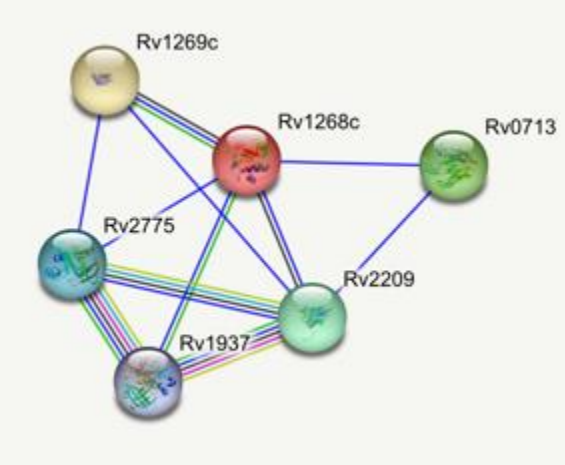


Figure D2: String analysis of *Mtb* Rv1268c and its predicted protein-protein interactions

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