# Construction of world's first *Mycobacterium tuberculosis* cytochrome P450 monooxygenase library

By

## Ipeleng Kopano Rosinah Kgosiemang

Dissertation submitted in fulfilment of the requirements of the degree

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**Promoter: Prof Khajamohiddin Syed**

**Co-Promoter: Prof Samson Sitheni Mashele**

# DECLARATION

I, **IPELENG KOPANO ROSINAH KGOSIEMANG** (SOUTH AFRICAN ID NUMBER: ████████ ), hereby certify that the dissertation submitted by me for the degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology (Free State). I hereby declare, that this research project has not been previously submitted before to any university or faculty for the attainment of any qualification. I further waive copyright of the dissertation in favour of the Central University of Technology (Free State).

I also state that expression vector modified/generated in this study is in collaboration with my doctoral colleagues, **Mr MOHAMMAD PARVEZ** (student number: ████████ ) and **Mr HANS DENIS BAMAL** (student number: ████████ ).

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**IPELENG KOPANO ROSINAH KGOSIEMANG**                              **DATE**

# Dedication

This thesis is dedicated to my late

Grandfather

**Martin Kgosiemang**

And

Aunty **Nthabiseng Kunene**

# ACKNOWLEDGEMENTS

KUNENE, for always having his home open for me and my laptop, and also encouraging me to pursue my dreams.

I would also like to thank, Kagiso XABA for providing me with unfailing support, continuous encouragement, and his patience throughout my years of study and through the process of research and writing this thesis.

Above all, I owe it to the Almighty God for granting me the wisdom, health and strength to undertake this research task and enabling me to its completion.

*"Believe in yourself! Have faith in your abilities! Without a humble but reasonable confidence in your own powers you cannot be successful or happy."*

**-Norman Vincent Peale**

# CONTENTS

# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| °C | Degree Celsius |
| AIDS | Acquired Immuno-Deficiency Syndrome |
| BCG | Bacillus Calmette-Guérin |
| bp | Base pair |
| CO | Carbon monoxide |
| CYP or P450 | Cytochrome P450 monooxygenase |
| cYY | Cyclo-L-Tyr-L-Tyr |
| DNA | Deoxyribo Nucleic Acid |
| EMB | Ethambutol |
| *et al*. | *Et alia* (and others) |
| FAD | Flavin Adenine Dinucleotide |
| Fe (II) | Iron (II) oxide |
| Fe (III) | Iron (III) oxide |
| FP | Forward primer |
| FQ | Flouroquinolone |
| HIV | Human immunodeficiency virus |
| ID | Identity |
| IDT | Integrated DNA technology |
| INH | Isoniazid |
| KatG | Catalase peroxidase |

| | |
|---|---|
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LAM | Lipoarabinomannan |
| LB | Luria-Bertani |
| *M. africanum* | *Mycobacterium africanum* |
| *M. bovis* | *Mycobacterium bovis,* |
| *M. canetti* | *Mycobacterium canetti,* |
| *M. caprae* | *Mycobacterium caprae,* |
| *M. microti* | *Mycobacterium microti.* |
| *M. pinnipedii* | *Mycobacterium pinnipedii* |
| m-AGP | Mycolyl-arabinogalactan-peptidoglycan |
| MCS | Multiple Cloning Site |
| MDR-TB | Multi-Drug Resistant Tuberculosis |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NEB | New England Biolabs |
| P450cam | CYP101A1 from *Pseudomonas putida* |
| PCR | Polymerase chain reaction |
| pDRAW | DNA analysis software |
| PZA | Pyrazinamide |
| RH | Substrate |
| RIF | Rifampicin |

| | |
|---|---|
| RNA | Ribonucleic acid |
| R-OH | Hydroxylated product |
| RP | Reverse primer |
| rpm | Revolutions per minute |
| sp. | Species |
| STI | Sexually Transmitted Infection |
| TB | Tuberculosis |
| TDR | Totally drug-resistant |
| $T_m$ | Melting temperature |
| US | United States (of America) |
| WHO | World Health Organisation |
| WWW | World Wide Web |
| XDR-TB | Extensively or extremely drug-resistant Tuberculosis |

# LIST OF FIGURES

# LIST OF TABLES

# CHAPTER 1

# ABSTRACT

The actinomycete *Mycobacterium tuberculosis* causes Tuberculosis (TB), a chronic lung disease in humans and continues to be one of the greatest threats to mankind. Large number of studies showed that *M. tuberculosis* cytochrome P450 monooxygenases (P450s) can be used as novel drug target. P450s are mixed function oxidoreductases well known for their role in essential cellular anabolic and catabolic processes. Despite the greater importance of *M. tuberculosis* P450s as novel drug targets, only four *M. tuberculosis* P450s (apart from highly conserved CYP51) have been functionally characterized for their *in vivo* role. The major challenges in *M. tuberculosis* P450 research is expression of *M. tuberculosis* P450s and identification of substrate(s).

This study is aimed to develop world's first *M. tuberculosis* P450 expression library by cloning remaining 14 *M. tuberculosis* P450s. In order to clone 14 *M. tuberculosis* P450s a cloning strategy was developed such that all 14 *M. tuberculosis* P450s was cloned into expression vector. In this study, expression vector pINK_A was modified in its multiple cloning site by incorporating more restriction enzyme sites to accommodate all 14 *M. tuberculosis* P450s. The modified vector was named as pINK_d. Restriction profiling of 14 *M. tuberculosis* P450s were carried out and suitable restriction enzymes were selected for directional cloning of *M. tuberculosis* P450s into pINK_d vector. The pINK_d and 14 *M. tuberculosis* P450 cDNAs were synthesized and all synthesized P450 cDNA were subsequently cloned into pINK_d. The plasmid DNA clones (or constructs) containing *M. tuberculosis* P450 cDNA was transformed into *E. coli* DH5α cells and the recombinant *E. coli* cells were selected on Luria-Bertani agar plates containing ampicillin antibiotic. Plasmids from the recombinant cells were isolated and subjected to restriction enzyme

digestion analysis. Restriction enzyme digestion analysis of plasmids revealed that all 14 *M. tuberculosis* P450s were successfully cloned as correct size of cDNA corresponding to respective *M. tuberculosis* P450s was released upon digestion with restriction enzymes. This study will pave the way for expression and characterization of *M. tuberculosis* P450s. Thus developing *M. tuberculosis* P450 based novel anti-TB drugs. The *M. tuberculosis* P450 *E. coli* library developed in this study will be patented after confirming *M. tuberculosis* P450s expression.

Apart from my Masters study, I also supervised two B. Tech student projects and managed to publish an article with students. Furthermore, I also worked on a few other bioinformatics projects and earned co-authorship. Most of my research articles are published in high impact factor journals. The following is a list of my research articles:

1.   IKR Kgosiemang (co-author) (2016) Molecular evolutionary dynamics of cytochrome P450 monooxygenases across kingdoms: Special focus on mycobacterial P450s. Scientific Reports 6, Article number: 33099.

2. IKR Kgosiemang (co-author) (2015). Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes. Scientific Reports 5, Article number: 11572.

3. NT Mthakathi, IKR Kgosiemang et al, (2015). Cytochrome P450 monooxygenase analysis in free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A. Alage 30(3):233-239.

In addition to the above credits, I was featured on national TV and in newspapers for discovering a novel drug target. I also presented work at both national and international (Canada) conferences.

# CHAPTER 2

# INTRODUCTION AND LITERATURE REVIEW

## 2.1. Introduction

### 2.1.1. History of tuberculosis

Regardless of any vaccination or strong drugs used, tuberculosis (TB) remains one of the oldest human diseases and one of the biggest killers among infectious diseases worldwide (McEvoy *et al*., 2009). It causes morbidity and mortality among the world's poor. This airborne bacterial disease affects 8 to 10 million people living in developing countries and 2 to 3 million of them die from this disease each year (Coovadia and Benatar, 1991).

TB is regarded as a major threat in Africa owing to its high incidence of infection. In most people, it remains dormant and never gives rise to the disease, but can later explode in response to low immunity (Coovadia and Benatar, 1991). TB is one of the most prevalent diseases and it has been widespread over an extensive period, but even though it is contagious, it is far less of an obvious threat than other dramatic infectious epidemics such as plague, smallpox and cholera, which over the years have caused a great degree of public alarm (Coovadia and Benatar, 1991). As a result, TB has never caused any public alarm, even though it has caused more deaths than the above-mentioned epidemics (Coovadia and Benatar, 1991).

Previously, medical writers did not consider TB a serious infectious disease and therefore took it for granted. For example, Creighton, who published "A History of Epidemics in Britain" (Creighton, 1891) did not even consider TB an infectious disease (Figure 2.1). The ignorance came back to bite them by starting to reveal itself in Europe

between the seventeenth century and nineteenth century (Dubos and Dubos, 1952; Benatar, 1991), when 20 percent of the population living there perished from the epidemic (Benatar, 1991). Doctors in Europe were even scared to conduct research on finding a cure because the disease was so fatal. During the last half of the nineteenth century, the spread of TB decreased in response to improved sanitation and housing (Dubos and Dubos, 1952; Packard, 1991). In Britain, TB reached its peak in the late eighteenth century; at that time the annual death rate was about 500 per 100 000 of the population. This started to change and the numbers dropped to 200 per 100 000 of the population by 1882, when Robert Koch discovered the tubercle bacillus, and to 50 per 100 000 of the population just before anti-tuberculosis drugs began to be used in the 1940s and then down to approximately 5 per 100 000 per year (Mckeown, 1979).



**Figure 2.1. Creighton Charles** - One of those who did not regard tuberculosis as an infectious disease; author of "A History of Epidemics in Britain" (Underwood, 1948).

The earliest evidence of TB in humans was found in the vertebrae of Stone Age man and in records of ancient Greece, Rome, India, China and medieval Europe, as well as Egyptian mummies (Glatthaar, 1991). About 5 500 years ago, plenty of evidence of Pott's

4

disease was found in Egyptian mummies, but early workers concluded that all the infections where caused by *Mycobacterium bovis* (Cave and Demonstrator , 1939; Morse *et al.*, 1964; Morse, 1967). There is, however, solid evidence from amplified DNA recovered from mummies that *M. tuberculosis* (*Mtb*) was the cause of the disease in ancient Egyptians (Figure 2.2) (Nerlich *et al.*, 1997; Crubézy *et al.*, 1998).



**Figure 2.2. An Egyptian mummy**. It was believed that this 2,600-year-old Egyptian woman died from ovarian cancer; however, investigations revealed that she died from tuberculosis (A Blog About History, 2009).

As people roamed around moving from one continent to another, the movement not only led to the rapid growth of urban centres and conditions favouring the development of TB, but also a change in the composition of people at risk (Metcalf, 1991). Before the end of the nineteenth century, TB was an uncommon disease in South Africa. The first time South Africa encountered this epidemic was when immigrants from other countries migrated to South Africa; people from South Africa had no knowledge about this disease. Most immigrants at that time were resistant to the disease because of a long period of exposure to

TB (Glatthaar, 1991). Black people were not resistant at that time; as a result, the disease started spreading very fast.

The reasons for the rapid spread were that there were many mines and miners working there (Lyons, 1991). Miners who contacted TB spread the infection, especially since their working conditions were conducive to the spread: ventilation in the shafts was poor and humidity was very high, the mineworkers used to work long hours and they were working very close to one another (Figure 2.3).



**Figure 2.3. Health-endangered miners**. These miners are working very close to one another and they spend a lot of time together underground, therefore this may lead to the spread of infectious disease epidemics, especially TB (Basu *et al*., 2009).

Industrialization also led to many people moving to the same area for the sake of jobs, where they lived in industrial shantytowns. The houses were dark because they did not have any windows and the poor ventilation favoured the spread of TB (Freedman, 1966). The history of TB is not just the history of an infectious disease, but is a history that brings nations together, changes the way people think, and also changes environmental conditions.

### 2.1.2. Impact of TB in the world

This airborne bacterial disease remains the leading cause of death worldwide. At present, approximately one-third of the world's population is infected by latent TB, which can later develop into active TB (WHO, 1994). The World Health Organization (WHO) reported that in 2013, TB infected 9 million people and 1.5 million deaths were reported (WHO, 2016). According to the 2014 WHO statistics, 9.6 million people fell ill with TB and 1.5 million of them died from the disease (WHO, 2016). Between 2000 and 2014, the number of people affected by TB declined every year, even though the decline was very slow. Therefore, the government, private health facilitators, public health facilitators, patients, and the community have designed a strategy to work together to reach the 2020-2035 goal of ending the global TB epidemic (Table 2.1) (WHO, 2016). TB does not affect only some areas; it affects the whole world, even though developed countries are in a better situation than developing ones. In 2014, South Eastern Asia and the Western Pacific regions had an increase of 56% in new cases of TB (WHO, 2014). However, Africa was the worst affected region, with 280 cases per 100 000 of the population in 2013.

**Table 2.1. The end TB strategy** (WHO, 2015).

| Indicators | MILESTONES | | TARGETS | |
|---|---|---|---|---|
| **Year** | 2020 | 2025 | 2030 | END TB 2035 |
| **Reduction in number of TB deaths compared with 2015 (%)** | 35% | 75% | 90% | 95% |

| Reduction in TB incidence rate compared with 2015 (%) | 20% (<85/100 000) | 50% (<55/100 000) | 80% (<20/100 000) | 90% (<10/100 000) |
|---|---|---|---|---|
| TB-affected families facing catastrophic costs due to TB (%) | Zero | Zero | Zero | Zero |

This is a strategy to end the global TB epidemic, with the aim of preventing and controlling the disease and taking good care of people living with TB, starting from 2015. The main aim of this project is to reduce the incidence of TB by 2% in 2015 and by 10% per year by 2025. The other plan is to reduce the rate of motility by 15% in 2015 and by 21.5% by 2025. This project is planned to run from 2015 to 2035 (WHO, 2015).

South Africa remains one of the countries with the highest burden of TB, with an estimate of 450 000 cases of active TB in 2013 (WHO, 2014). This means that almost 1% of the population of about 50 million develop active TB each year. This makes the incidence in South Africa, which has increased over the past 15 years, the third highest country after India and China (SANAC, 2011).

The WHO has estimated that in about 270 000 of the 450 000 cases in South Africa, people have both HIV and TB infection; this is usually called TB and HIV co-infection. The latest report from the South African Department of Health states that 73% of TB patients are HIV positive (Annual Performance Plan 2014/15 - 2016/17, Department of Health, South Africa). The WHO figures and South African figure are normally different, since the WHO figures are estimates and the South African ones are the actual figures (SANAC, 2011).

People living with HIV/AIDS are more likely to develop active TB than people without HIV/AIDS, even if the latter have latent *M. tuberculosis*. The combination of HIV and TB is fatal, because each disease speeds up the progress of the other. Furthermore, HIV infection can speed up the progression from latent to active TB (Mayer and Hamilton, 2010).

### 2.1.3. TB: Types and symptoms

*M. tuberculosis* is resilient bacteria capable of affecting any organ of the human body. The respiratory system acts as the common form of transmission through the air by inhalation of droplet nucleic-containing organisms of the tubercle bacillus from an infected individual into the lungs (Cole *et al*., 2005). *M. tuberculosis* can also enter the body through the gastrointestinal tract. This is the usual portal entry for the bovine strain, which is spread by contaminated milk, and through open wounds in the skin, but these forms of entry are rare (Grange and Yates, 1994; Alzohairy, 2007). Most *M. tuberculosis* infections affect the pulmonary system and can destroy parts of the lungs, making it difficult to breathe. The remaining organs, which amount to a small percentage of approximately 25%, are considered secondary TB infection organs. The bacteria may spread to and damage other parts of the body, such as the digestive tract, the genitourinary system and the urogenital tract, in which the kidney is the most common site of this kind of TB. Bones and joints may also be affected; the patient will start experiencing pain, swelling of the joints and limitation of the range of movement and Pott's disease of the spine may develop, marked by spinal deformity and other bone defects (Figure 2.4). Other sites of infection may include the central nervous system, causing meningitis. Scrofula was common in the middle ages and presented itself with swelling of the lymph nodes in  the neck and the skin could be affected as well (Golden and Vikram, 2005; Nigg *et al*., 2008; Kim *et al*., 2009; Peto *et al*., 2009; Semba *et al*., 2010). These forms are called secondary TB and are far less common. The disease is characterized

by the development of granulomas or tubercles in infected tissues. Another type of TB if known as disseminated TB; this type of TB is normally known as miliary TB, which develops when one is in direct contact with a person who has the disease and is living in unhygienic conditions, with infections in either the lungs or any other organs of the body (Dugdale *et al.*, 2009). There are many ways in which TB can present itself, and its presentation usually depends on the age, gender or race of the patient. Irrespective of these factors, if the infection is left untreated for a long time, it is more likely that the patient will die from the disease.



**Figure 2.4. Presentation of tuberculosis in different areas of the human body**. Figure A shows magnetic resonance imaging of the central nervous system being attacked by *M. tuberculosis* causing CNS tuberculoma. Figure B illustrates the spine with intraosseous and epidural abscesses resulting in spinal canal stenosis known as Pott's disease. Figure C displays genitourinary tract TB, where the right kidney has been destroyed by lobar calcification in a renal TB patient (Rock *et al.,* 2008; Merchant *et al.*, 2013; Ansari *et al.*, 2013).

## 2.2. *M. tuberculosis* - The pathogen

### 2.2.1. Introduction

*M. tuberculosis* is the main cause of the virulent disease called TB, as described above. TB is an ancient disease with a history linked with the evolution and migration of mankind. It is believed that this causative pathogen evolved 3 million years ago (Gutierrez *et al.*, 2005). In the year 1882 Robert Koch first identified and described this bacterium, and got a Nobel Prize for his work.

*M. tuberculosis* belongs to the *M. tuberculosis* complex, which is defined as the original cause of TB in different hosts. Other TB organisms may include *M. bovis*, *M. africanum*, *M. canetti*, and *M. microti*. *M. caprae* and *M. pinnipedii* are considered to be variants of *M. bovis*. Previously, it was believed that *M. tuberculosis* evolved from *M. bovis* during the domestication of cattle (Stead *et al.*, 1995). However, genome sequencing of both species revealed that *M. bovis* has several DNA deletions while maintaining 99.95% identity with *M. tuberculosis* and no new genetic material supporting the opposite scenario was found (Brosch *et al.*, 2002). Successive DNA deletions of regions of difference resulted in the branching off members of the *M. tuberculosis* complex (Brosch *et al.*, 2002). These genetic analyses indicate that members of the *M. tuberculosis* complex are the clonal progeny of an ancestral strain of *M. canetti*, also referred to as *M. prototuberculosis* (Gutierrez *et al.*, 2005).

### 2.2.2. Characteristics of *M. tuberculosis*

Mycobacterium organisms are non-motile, non-spore forming, weakly gram-positive, acid-fast bacilli that appear straight or slightly curved under a microscope, with a length of 1 to 4 mm and width of 0.3 to 0.6 mm. *M. tuberculosis* belongs to the genus *Mycobacterium* and *Actinomycetales* order. The genus *Mycobacterium* shares this order with *Corynebacterium*, *Nocardia* and *Rhodococcus*. The fact that these bacteria fall into the same order as the

*Mycobacterium* means that they share some similarities. For example, these bacteria also express unique mycolic acids in their cell envelope (Barry *et al*., 1998). Mycobacteria are facultative intracellular bacteria that multiply within phagocytic cells, particularly macrophages and monocytes. Even though many mycobacterial species are environmental, *M*. *tuberculosis* is strictly parasitic. *M*. *tuberculosis* is a slow-growing pathogenic mycobacterial species, which can divide within 12 to 24 hours and be grown on an agar plate for up to 21 days. Proposed mechanisms for its slow growth include limitation of nutrient uptake through the impermeable cell wall and slow rates of ribonucleic acid (RNA) synthesis (Harshey and Ramakrishnan, 1977).

Mycobacteria possess a unique cell envelope structure made up of three layers. The first layer – the waxy exterior – also called the mycobacterial outer membrane is made up of porins, which are transport proteins. The second layer – the periplasmic cell wall – contains peptidoglycan, arabinogalactans and mycolic acids. The last layer – the cytoplasmic phospholipid bilayer – consists of alpha-1,4 glucan, arabinomannan and mannan, which are polysaccharides found on the exterior of the cell wall (Figure 2.5) (Daffé and Draper, 1997; Bhamidi *et al*., 2008). The cell envelope of mycobacteria makes the walls anti-permeable to various molecules, resistant to drying and resistant to drugs. Because of this cell envelope, the bacteria can survive under unfavourable conditions, as well as exhibit distinctive immune-stimulatory properties (Daffé and Draper, 1997). Mycolic acids make up approximately 40 percent of the outer membrane mass mycolic acids. The structure of the cell wall also comprises three covalently linked micromolecules, namely peptidoglycan, mycolic acid and arabinogalactan (Mcneil, 1996). Peptidoglycan, which forms the backbone of mycolyl-arabinogalactan-peptidoglycan (m-AGP), is the most important feature of the mycobacterial cell envelope. The phosphodiester bond covalently binds the peptidoglycan to mycolyl arabinogalactan (Brennan, 2003). It has been speculated that peptidoglycan is known

to be resistant to lysosome, and that the *N*-glycolyl group of the muramid acid residue in the peptidoglycan may protect the organism from degradation (Brennan, 2003). Most of the frame structure of the cell wall is made up of *m*-AGP (Brennan, 2003); the mycolic acid is clustered together forming tetra-mycolyl-penta-arabino-furanosyl clusters. The arabinon part of the cluster is glycosidically linked to a linear D-galactan forming a disaccharide-phosphate, which is in turn linked to the peptidoglycan (Brennan, 2003). The mycolic acid usually moves vertically to the peptidoglycan, and other glycolipids of the cell wall, such as lipoarabinomannan, will attach themselves to the mycolic acid and form a pseudo lipid bilayer (Brennan, 2003). Researchers have found that galactosamine was also part of the wall, but only in slow-growing mycobacteria (Brennan, 2003). This amino acid is found attached to arabinose of arabinogalactan in the cell wall skeleton and is reported to be a free amine (Draper *et al.,* 1997).
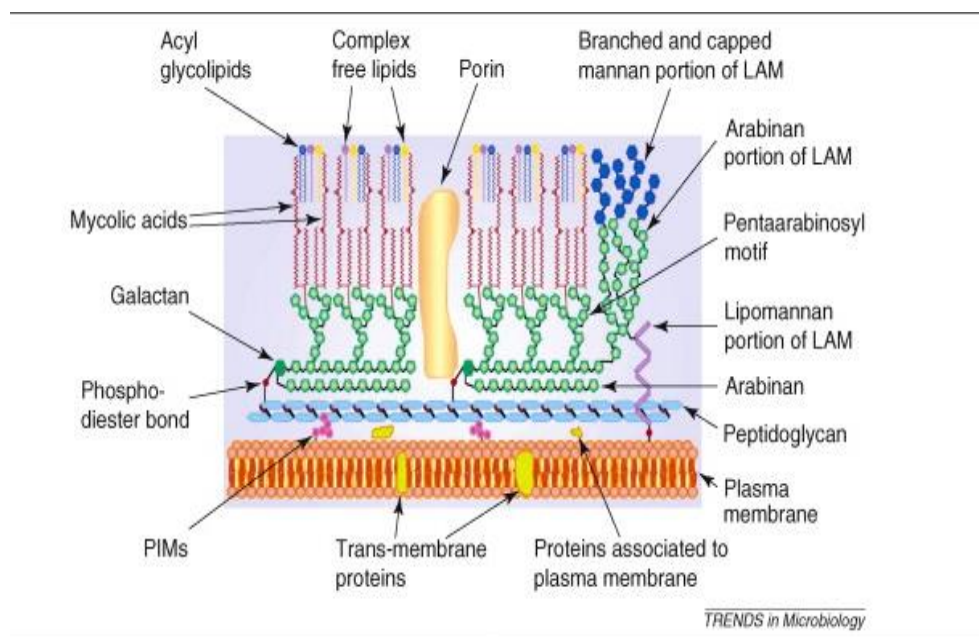


**Figure 2.5. Schematic representation of the mycobacterial cell wall (taken from Gokhale *et al.,* 2007)**. The mycobacteria envelope is made up of three plasma membrane layers, namely the waxy exterior; the peptidoglycan containing the unique mycolic acid and

arabinogalactan and the capsulated structure containing polysaccharides, proteins and lipids (Gokhale *et al*., 2007; Medjahed *et al*., 2010).

Chemically, the mycolyl arabinogalactan-peptidoglycan complex forms an integral part of the cell wall, and lipoarabinomannan (LAM) plays a role in the virulence and immune-pathogenesis of mycobacterial disease. The exterior wall has polar and non-polar lipids. These lipids are not directly bound to the structure; instead they are distributed on the cell surface. The distribution depends on the strain (Brennan, 2003). During initiation of infection, these proteins, lipids and lipoglycan are responsible for cell signalling, where the mycolyl-arabinogalactans-peptidoglycan complex determines the viability of the bacterial cell (Brennan, 2003).

The structure of the mycolic acid comprises large fatty acids, which are alpha-alkyl and beta-hydroxylated branched (Besra and Brennan, 1997). Mycolic acids are grouped into two families: the alpha-mycolates deoxygenated and mycolate oxygenated. *M*. *tuberculosis* makes three classes of mycolic acids, and these differ primarily from each other in terms of the presence and nature of the oxygen-containing substituents in the distal portion of the methoxy group adjacent to a methyl branch (Draper *et al*., 1997). On the arabinon portion, the mycolic acids present themselves as a tetra-mycolyl-pentaarabinofuranosyl cluster (Besra and Chatterjee, 1994).

### 2.2.3. Progression and infection

A few stages may take place during the progression of *M*. *tuberculosis* in the host. The lungs are the initial site of contact between the host and the organism, but other organs, such as the skin, oral cavity and gastrointestinal tract, may also initiate the entry of the organism (Bezuidenhout and Schnneider, 2009). During the early stage, the organism is inhaled into the

periphery of the lungs, and once it reaches the lungs it is phagocytised. This process initiates innate immunity to protect the host. The phagocytised organism is then transported to the hilar lymph nodes and presented to the T cells. Once the T cells have been presented with the phagocytised organism, this stimulates the activation, proliferation and differentiation of the T cells. If the organism survives, the process of adaptive immunity takes control and a delayed type hypersensitivity response continues at the primary site of infection (Bezuidenhout and Schnneider, 2009).

In the beginning little reaction will be taking place and small numbers of neutrophils will be seen surrounding the organism. After a few days, the macrophage, which usually cleans the cell, will come and collect the organism, trying to get rid of the infection. If the eradication does not go well, granuloma will start forming. These granulomas form when the macrophage starts forming microscopic aggregates that may end up changing the normal structure of the lungs. They do this by becoming active and starting to develop into epithelioid cells, gaining abundant pale-pink cytoplasm with indistinct cell borders, a vesicular nucleus and often a distinct nucleolus. These changes show the changes from a phagocytic to a secondary function that improves the micro-biocidal properties of macrophages, but can on the other hand unfortunately induce necrosis of lung tissue. These macrophages may fuse together and start forming multinucleated giant cells. The T-lymphocytes will be found scattered and surrounding the macrophages. The macrophage, the giant cells and lymphocytes are collectively known as granuloma (Bezuidenhout and Schnneider, 2009).

A few weeks later, the granuloma can be seen under a microscope; it will appear grey but as it enlarges it has a yellow colour. On close examination, necrosis will be seen in the centre of the granuloma. Adjacent granulomas will also undergo necrosis, causing extreme

tissue damage. Necrosis of the central portion of the lesion results in a relatively solid cheesy appearance called caseous necrosis (Bezuidenhout and Schnneider, 2009).

The formation of the granuloma occurs in the peripheral part of the lungs near the pleura. Typically, this lesion is called the Ghon focus, and the combination of regional lymph nodes' involvement and primary lesion is called the Ghon complex (Hart, 1997; Husain *et al.*, 2005). Another response that may occur at the site of the necrosis area is liquefaction, with the liquid material sloughing into a connecting bronchus and producing a cavity. The tubercular material sloughed from the walls of the cavity enters the tracheobronchial tree, causing damage to the lungs. If this is not taken care of, small cavities close and leave fibrous scars. As inflammation heals, scarring may narrow or close the bronchial lumen near the junction of the cavity and the bronchus. The gaseous material may thicken and be unable to flow through the communicating channel, so the cavity fills with this material and the lesion becomes similar to the sloughed encapsulated lesion. The lesion may remain in an inactive stage for long periods or regenerate its bronchial communication and be the site of active inflammation (Bezuidenhout and Schnneider, 2009).

The disease may spread through the lymphatics or blood vessels. The organisms that pass through the lymph nodes reach the bloodstream in small numbers and may initiate occasional lesions in various organs. This dispersal of the organisms is referred to as lymphohematogenous dissemination. Hematogenous dissemination, an acute phenomenon, usually gives rise to miliary TB; it occurs when necrosis focus erodes a blood vessel, allowing large numbers of organisms to enter the vascular system and be disseminated to various organs (Bezuidenhout and Schnneider, 2009).

TB can present itself in two forms, and the way the immune system responds to these conditions depends on whether it is active or latent TB. Two million people in the world are

estimated to be infected by latent TB. This large number represents an enormous reservoir of potential reactivation TB, which can spread to other people (Lin and Flynn, 2010). During this phase, the organism enters the host body and then the immune system reacts by building a wall around the organism. TB can remain alive for a long time in these walls without being active; this means that the bacteria cannot be spread to the next person or even affect the host (www.cpmc.columbia.edu). An active form is considered to be a disease. People with a weak immune system, drug and alcohol abusers and very ill people are more susceptible to this kind of TB. Shortly after the active organism enters the host of a weak immune system, the disease will start damaging the body tissues and organs. When latent TB becomes active, the bacterium breaks out of the walls and begins to multiply rapidly (www.cpmc.columbia.edu). Once active TB breaks out, the infected person will start showing symptoms of pulmonary TB, coughing a lot and exhibiting blood in their sputum, fever, tiredness and weight loss. However, active TB can be treated effectively by taking several different medications to reduce cases of mortality (WHO, 2014).

### 2.2.4. Disease prevention and treatment

The cell wall component of *M. tuberculosis* has shown more importance than ever in the development of new drugs and possible vaccines (Draper *et al*, 1997). This unique complex structure represents the best target for novel anti-mycobacterium agent. Most diseases can be prevented by using specific vaccines, but TB is one of the diseases for which Bacillus Calmette-Guerin (BCG), which is an *M. bovis* strain, is used as a vaccine (Wang and Xing, 2002). BCG is the only vaccine against TB known to man. When a child is born it is vaccinated with BCG for the prevention of TB. BCG can help reduce the incidence of TB during childhood, particularly the form causing meningitis, but it is not very effective against adult TB and does not prevent infection with the organism (Flynn, 2004). It is possible for a

person who has been immunised with BCG to be infected with TB and also to be prone to active TB. It has been reported that during human trials the effectiveness of BCG differed from 0-80% (Flynn, 2004). Despite researchers thinking that they have a vaccine against TB, much work and research clearly needs to be done to develop more effective vaccines against TB. Many studies on TB vaccine have been conducted, but none of them has been successful.

As mentioned before, *M. tuberculosis* remains a serious problem worldwide. This disease has worsened, especially in developing countries, because of lack of chemotherapeutic agents, lack of knowledge and lack of resources. As a result, the world is facing an era of drug resistance. This has brought about great stress and problems for governments, because of the increasing spread of TB. In 2013, approximately 480 000 people developed multidrug-resistant TB (MDR-TB) worldwide (WHO, 2014). More than half of these cases occurred in India, China and the Russian Alliance. MDR-TB is a kind of TB caused by bacteria that do not respond to at least first-line anti-TB drugs (WHO, 2014). MDR-TB is primarily caused by incorrect use of anti-TB drugs and the use of poor quality medicines (WHO, 2O14). However, MDR-TB can be treated and cured by using second-line drugs (WHO, 2014). However, second-line treatment options are limited and the recommended medicines are not always available. The extensive chemotherapy that is required is more expensive and can produce severe drug reactions in patients (WHO, 2014). In some cases more severe drug resistance can develop, known as extensively drug-resistant TB (XDR-TB). XDR-TB is a form of multi-drug-resistant TB that responds to even fewer available medicines, including the most effective second-line anti-TB drugs (WHO, 2014).

The problem of drug resistance has been there for a long time; it is not a new development. It started after the use of Streptomycin as a treatment for TB in 1944, when the *M. tuberculosis* strain started showing resistance to this drug (Ryan, 1992). Spontaneous

chromosomal mutation in the genes causes drug resistance (Zhang and Yew, 2009). The problem of drug resistance arises from failure of the patient to comply with treatment, monotherapy due to irregular drug supply, doctors not knowing what they are treating, and also transmission of the resistant strain from an infected person to another person who does not have TB at all or a person who has TB but not the resistant TB strain (Vareldzis *et al.*, 1994).

Many drugs can be used for the treatment of TB, but it seems as if these drugs have been unable to skip the drug-resistant era. Isoniazid (INH) is one of the drugs used as anti-TB drugs and is very common among the other known TB drugs. INH was first discovered in 1952 (Zhang and Yew, 2009). INH is considered to be the most frequently used first-line drug against TB. This drug is effective in treating TB disease and latent infection, but it cannot treat active TB. INH works best on growing tubercle bacilli and under aerobic conditions (Zhang and Yew, 2009). This prodrug is catalysed by the catalase peroxidase enzyme, which is encoded by the katG gene to produce highly reactive species, which will then attack many obstacles in *M. tuberculosis* (Zhang *et al.*, 1992; Telenti, 1998). The enoyl-acyl (InhA) enzyme, which is responsible for the elongation of the fatty acid in mycolic acid synthesis, is the first target and will be inhibited by INH, preventing formation of this complex (Banerjee *et al.*, 1994). The other drug used is rifampicin (RMP), which was discovered in 1966 (Zhang and Yew, 2009). RMP falls under first-line drugs and it is one of the most important drugs used against TB. Like INH, RMP also destroys other cell wall components that induce TB. Unlike INH, RMP is active against both growing and non-replicating bacilli with low metabolic activity (Zhang and Yew, 2009). RMP and INH are considered the short course treatment regime for TB, because of their effective antimicrobial action (Rattan and Ahmad, 1998). These drugs have shown high sterility activity *in vivo*, and it can shorten the time of treatment from 12-18 months to 9 months (Mitchison, 1985). Their

mechanism of action is to inhibit the RNA synthesis. They do this by binding to the beta subunit of the RNA polymerase (Zhang and Yew, 2009). This binding prevents the initiation of RNA synthesis and as a result inhibits the process of transcription (Janin, 2007). Pyrazinamide (PZA) is also an important first-line drug, which was discovered in 1952 (Zhang and Yew, 2009). This drug works best when used in combination with INH and RMP. It functions best in an acidic pH environment and in anaerobic conditions, where it is able to inhibit persistent bacilli that were not inhibited by other drugs such as INH and RMP. PZA is important because it can reduce the treatment time from 9-12 months to 6 months (Mitchison, 1985).

Another anti-mycobacterial drug used is ethambutol (EMB), which was first discovered in 1961 (Zhang and Yew, 2009). It is a first-line drug used with INH, RMP and PZA. This is used to prevent the emergence of drug resistance (Zhang and Yew, 2009). EMB works best on growing bacilli and not on non-replicating bacilli. It inhibits the biosynthesis of arabinose, which plays an important role in the cell wall's arabinogalactan (Takayama and Kilburn., 1989). *M. tuberculosis* contains enzymes, and these enzymes are the target of EMB. These enzymes include embA, embB and embC. The embB is responsible for the production of arabinogalactan. As a result, the EMB drug targets this embB enzyme (Lety *et al.*, 1997; McLean and Munro, 2008). Flouroquinolone (FQ), on the other hand, is used as a novel therapeutic drug against TB. FQ is classified as a second-line drug and is used for the treatment of MDR-TB (Field *et al.*, 2012). FQ is known for its cruel inhibition of DNA gyraseA and gyraseB. It does this by interfering with bacterial chromosomal replication, DNA recombination and transcription (Field *et al.*, 2012). These drugs play an important role in disrupting the cell wall and enzyme production important for the functioning of this cell wall.

## 2.3. Cytochrome P450 monooxygenases

## 2.3.1. Introduction

Cytochrome P450 monooxygenases (CYPs/P450s) are heme-thiolate proteins found in all life forms from prokaryotes (archaea and bacteria), lower eukaryotes (fungi and insects) to higher eukaryotes (plants and animals, including humans) (Nelson, 2013). Cytochrome P450 superfamily consists of more than 12 456 members joined into over 1000 families (Nelson, 2011). The P450s are divided into two groups: those that metabolise xenobiotic (drugs, pollutants, agrochemicals, etc.) and those that play important roles in diverse physiological processes including steroid and cholesterol biosynthesis, fatty acid metabolism (prostacyclin, thromboxane) and the maintenance of calcium homoeostasis (McKinnon *et al*., 2008; Kelly and Kelly, 2013). The families from the first group metabolise a broad variety of different structures; biosynthesis of these P450s is often substrate-inducible and their gene knockout sometimes does not show any obvious phenotype (Gonzalez and Kimura 2003). The P450s from the second group have narrow substrate specificity. Recently P450s were classified into different groups based on their substrate class (Parvez *et al*., 2016). The main substrate class includes terpenes, lignin constituents, fatty acids, steroids, alkanes, vitamins and eicosanoids (Parvez *et al*., 2016). The general reaction of monooxygenation of substrates by P450s is shown below (Bernhardt, 2006):

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$
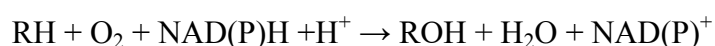
At first P450s were described as mixed-function oxidases and then in the early 1950s as mono-oxygenases (Mason *et al*., 1955; Hayaishi *et al*., 1957). Omura and Sato then came up with the name 'cytochrome P450', which described the coloured substance that was found in a cell. All of this happened in 1962 (Omura and Sato, 1962). This coloured substance produced an uncommon absorption peak at a wavelength of 450 nm when reduced and bound

with carbon monoxide in rat liver microsomes (McKinnon *et al.,* 2008). There was a contradiction in the name 'cytochrome'; some people called it an enzyme instead of a true cytochrome. The initial meaning of the term 'cytochrome P450' was coloured substance in the cell (Omura and Sato, 1962). Regardless of this, the name 'cytochrome P450' remained the same and is so widely accepted that any change would be impractical (McKinnon *et al.,* 2008).

The P450 gene superfamily can be widely divided into four classes based on mode of delivery for the electrons from nicotinamide adenine dinucleotide (NADH) (Prasad and Mitra, 2004). In the first class there are two electron transfer proteins, namely flavin adenine dinucleotide (FAD), which contains a reductase-iron sulphur protein-P450, in which the bacteria or mitochondria receive their NADH derivatives from the two-protein redox chain (Prasad and Mitra, 2004). Members of the second class only need one electron transfer protein, which is a reductase containing both FAD and flavin mononucleotide (Prasad and Mitra, 2004). These enzymes can metabolise xenobiotics and are found in the endoplasmic reticulum of eukaryotes. The third and fourth classes do not require any electron transfer partners. The enzymes that are found in the third class are thromboxane synthase and allen oxide synthase, which act on peroxy fatty acids (Prasad and Mitra, 2004). The fourth class directly binds and transfers electrons from reduced pyridine nucleotide to the heme iron (Prasad and Mitra, 2004).

It is not very easy to understand the structure-function relationship of such a complicated family of proteins. The only way to understand this is by comparing the crystal structures of numerous family members (Hasemann *et al.,* 1995). The important part of this study is the conserved functional significance of a particular region in the protein, irrespective of the divergence of the amino acid sequences (Hasemann *et al.,* 1995).

## 2.3.2.  Structure of P450

In order to understand the functions and properties of P450 enzymes, it is very important to know the structure and catalytic mechanism of these enzymes. *Pseudomonas putida* CYP101 of class I was the first structure of P450s to be deduced in 1985 (Poulos *et al.*, 1985). It took a long time before other classes could be explained, but at least the crystal structures of some P450s were reported. These include *Pseudomonad* CYP108, *Sachaaopolyspora erythraea* CYP107F, *Fusarium oxysporum* CYP55A1, rabbit P450 2C5, the famous *M. tuberculosis* P450 CYP51, thermophilic Archaeon *Sulfolobus solfactaricus* and other P450s (Poulos *et al.*, 1985; Ravichandran *et al.*, 1993; Hasemann *et al.*, 1994; Palma *et al.*, 1994; Cupp-Vickery and Poulos, 1995; Park *et al.*, 1997; Williams *et al.*, 2000; Podust *et al.*, 2001). All these structures give researchers a clear pattern for modeling other mammalian P450s, which assist them in drug-binding studies (Ortiz de Montellano, 1995). P450s have the same structure, even though they have different levels of amino acid sequence similarity. These proteins are spherical in shape and placed in such a way that they form a fold called the P450 fold (Sirim *et al.*, 2010). P450s contain a heme cofactor known as iron-protoporphyrin IX; the hemoprotein name comes from the heme cofactor. The heme group is found deep inside the protein, where its iron is coordinated to the cysteine thiolate group and a solvent molecule in the substrate-free state accompanies the sixth iron coordination site. This coordination arrangement gives P450 enzymes their unique spectral and catalytic properties. The heme group represents the active centre for catalysis (Schneider *et al.*, 2007). The active centre can accommodate different carbon-rich molecules, pressing them against the active oxygen atom.

The binding of carbon monoxide (CO) to sodium dithionite-reduced ferrous may cause an alteration of absorbance in the heme group to a maximum spectrum of 450 nm (Klingenberg, 1958; Omura and Sato, 1964). The binding of CO not only causes alteration, it

also hinders binding and activation of oxygen, stopping the normal activities of P450s from continuing. However, inhibition of P450 activities together with CO binding can be reversed by applying maximum efficient light at 450 nm. When other substrates, ligands and inhibitors bind to this P450 ferrous, they also cause an absorbance shift of the Soret peak in P450. These kinds of bindings can be observed by using a differential spectrophotometry (Jefcoate, 1978). For example, substrates that shift the sixth coordinated solvent (water molecule) in the ferric state of P450 normally make a spectral shift to a hypsochromic shift, revealing a low-to-high spin transition of the iron (Werck-Reichhart and Feyereisen, 2000).

### 2.3.3. Naming of P450s

The discovery of P450s in different laboratories opened doors in the research field in the 1970s and 1980s. When the P450s were identified, they had to be named, so at that time naming of P450s was done based on what was known about the P450 that had been identified, either by their molecular weight or preference for substrate (McKinnon *et al.*, 2008). However, this naming method brought about a lot of confusion and was faulty. Consequently, Nebert and his colleagues (Nebert *et al.*, 1987) formulated a standard nomenclature for the CYP450 gene family. This standard nomenclature was based on amino acid sequence comparisons and the evolutionary relationships of the corresponding genes (Nebert *et al.*, 1987). It was agreed that the nomenclature would be updated every time a new P450 was identified. The committee even had an official website based at the University of Memphis, USA (Nelson, 2006).

It is recommended that the naming of P450s should include the root symbol *CYP* representing cytochrome, an Arabic number describing the P450 family, a letter specifying the subfamily when two or more subfamilies are known to exist within that family and an Arabic number representing the individual gene (Nebert *et al.,* 1991). It is recommended that

when a P450 gene is being described, all letters and numbers should be written in italics (*CYP1A1*); however, this is different when describing a P450 enzyme. For a P450 enzyme the letters and numbers are written in non-italic style (CYP1A1). Naming of the P450 gene is not very complicated. The protein sequence and its representative sequence are aligned together from each family and subfamily, and this results in percentage identity. From the percentage results, there will be gaps, overlapping sequences and unmatched ends. The gaps and unmatched ends will not be counted in the overall length; however, the overlapping sequences will be compared and counted. If the sequence identity percentage is less than 40%, then that sequence starts the new member of a new family, but if the sequence is at least 40% or more identical to the other sequences, then that sequence belongs to the same family. When it comes to assigning subfamilies, the standard nomenclature rule of 55% identity is used. If the sequence identity percentage is less than 55%, then that sequence starts a new subfamily, but if the sequence identity percentage is 55% or more than the other sequence, that sequence belongs to the same subfamily as the other sequence.

### 2.3.4. Catalytic cycle of P450s

Cytochrome P450s form a very large superfamily of heme protein monooxygenases with wide distribution in most living organisms (Nelson *et al.,* 1996; Nelson, 2013; Parvez *et al.*, 2016). P450s are able to catalyse a number of reactions; however, the most common reaction catalysis by P450 is the monooxygenase reaction (Ortiz de Montellano, 1995). In 1968 researchers introduced the idea of creating an enzymatic cycle as a general process for P450 monooxygenation (Denisov *et al.*, 2005). This cycle is still used. The catalytic cycle needs two electrons and two protons; the two electrons will be delivered by NAD(P)H from one of the redox partners and the two protons contributed by bulk solvent. Monooxygenase enzyme catalyses the reduction of molecular oxygen by inserting one oxygen atom into a substrate,

while the second oxygen atom results in the formation of water as a side product (Denisov *et al.*, 2005).

The catalytic sequence of P450s is divided into four steps, as shown in Figure 2.6. The first is substrate binding, where the resting state of the P450 enzyme is an Fe (III) protein in a low spin state with cysteine thiolate on the firth axial position and water on the sixth axial position (Ortiz de Montellano, 1995). The binding of the substrate results in the removal of water, forming a five-coordinated ferric high-spin species. The second step is reduction of the substrate-heme protein complex to a ferrous state. During this phase, substrate binding causes a change in the redox potential *via* change in the structure of the porphyrin ring (Ortiz de Montellano, 1995). The third is binding of molecular oxygen to Fe (II) species. This phase is followed by reduction of oxygen to superoxide, $O_2$ and oxidation of Fe (II) to Fe (III) (Prasad and Mitra, 2004). The last phase is a second reduction step resulting in activated oxygen species. During this phase a second reducing electron, which is similar to the first electron, is introduced into the picture and it produces oxygen in the peroxide oxidation state. Then the two protons split off the oxygen atom as water, forming an activated oxygen species. This continues to the oxygen radical rebound mechanism through which the enzyme finally returns to its normal low-spin resting state after the release of the product (Ortiz de Montellano, 1995; Werck-Reichhart and Feyereisen, 2000; Munro *et al.*, 2007).
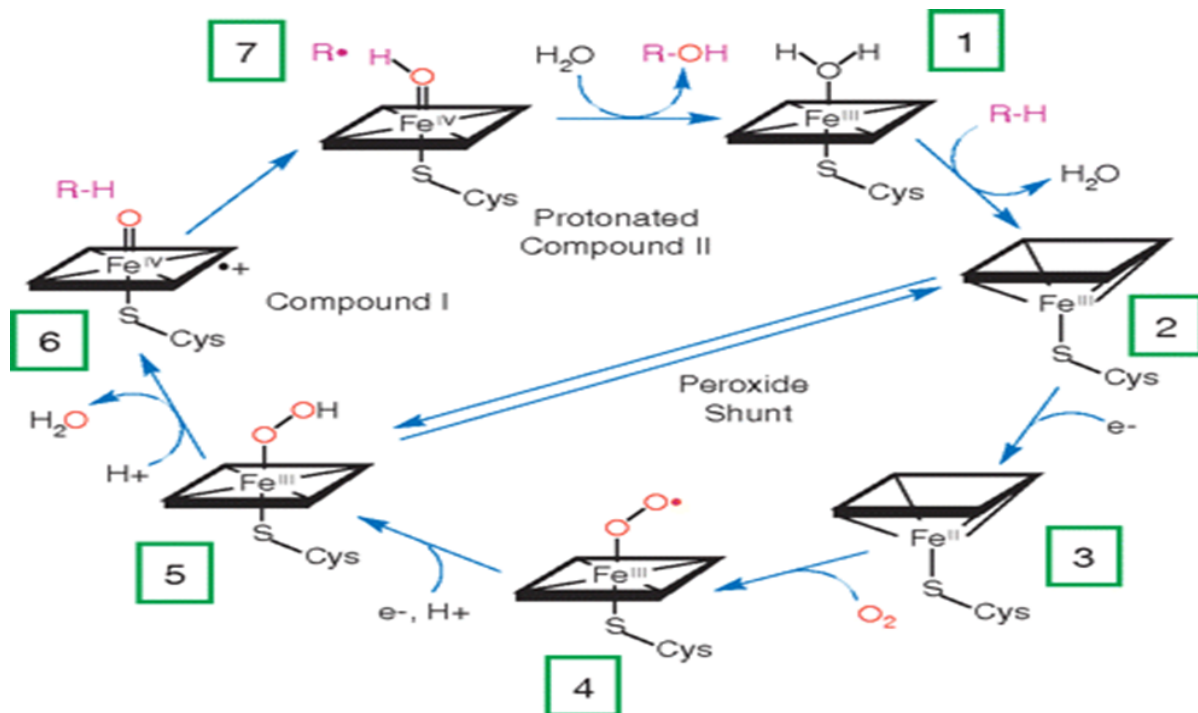
**Figure 2.6. Schematic representation of the P450 catalytic cycle and peroxide shunt pathway (taken from Krest *et al.,* 2013)**. The presentation illustrates catalytic cycle and all the structural changes that take place there. This figure also shows the reversible reaction of the peroxide shunt during the cycle.

**2.4. Problem statement**

**2.4.1.** *M. tuberculosis* **mechanism of drug resistance**

It has been reported that the *M. tuberculosis* strain is more resistant to INH than to any other drug. INH resistance is seen on the KatG S315T mutation and on the promoter region of mabA/InhA operon by causing overexpression of InhA (Banerjee *et al*., 1994; Rozwarski *et al*., 1998; Telenti, 1998; Hazbón *et al*., 2006). Resistance to RMP originates from a mutation in the rpoB gene that encodes the RNA polymerase subunit. This mutation may cause alteration to the drug-binding site (McLean and Munro, 2008). The main cause of resistance of PZA is the pncA mutation (Scorpio *et al.,* 1997). For EMB, resistance is due to a mutation in the embCAB operon, especially the embB, and sometimes on the embC (Telenti *et al.,*

1997). It is clear that mutation of some genes found on the cell wall of the *M. tuberculosis* strain is the main cause of drug resistance. However, this is not the only cause of bacterial resistance to drugs; other routes may include acquisition of gene plasmids and recombination of foreign DNA into the chromosomes (Guiney, 1984; Lacey, 1984; Johnsborg *et al.*, 2007).

### 2.4.2. P450s as novel drug targets against TB

The genome sequencing of *M. tuberculosis* revealed an uncommon occurrence of a large number of genes involved in lipid metabolism (Cole *et al.*, 1998). An interesting fact that became evident after genome sequencing of *M. tuberculosis* is that 20 P450s were found in this organism (Cole *et al.*, 1998). For a bacterial strain, this is a very high number, because bacteria normally have a low number of P450s. The high number of P450s found in *M. tuberculosis* shows that these P450s have great significance in bacterial viability because P450s make a good drug target and they may play a role in pathogenicity (Ouellet *et al.*, 2010; Hudson *et al.*, 2012). Most of the P450s found in *M. tuberculosis* were found to be taking part in cholesterol metabolism (McLean *et al.*, 2009). There are two important genes that play a role in *M. tuberculosis* pathogenicity; these are the products of cholesterol substrate, even though *M. tuberculosis* does not have a *de novo* pathway for sterol synthesis (Cole *et al.*, 1998; McLean *et al.*, 2009; Driscoll *et al.*, 2010). Azole drugs are documented to be antifungal drugs because of their high affinity to P450 proteins, making them good candidates for anti-TB drugs (Munro *et al.*, 2003). There are numerous possible major *M. tuberculosis* P450 drug targets, namely CYP121, CYP125, CYP128, CYP142 and CYP51 (Hudson *et al.*, 2012). Among these CYP isoforms, CYP121, CYP125 and CPY128 are the most promising anti-tubercular drug targets (Hudson *et al.*, 2012) and recent studies suggested that *M. tuberculosis* P450s particularly CYP121 and CYP141 are highly conserved at protein level compared to other P450s from different biological kingdoms (Parvez *et al.*,

2016). Furthermore, CYP121 and CYP141 P450s were reported to serve as diagnostic markers for identification of *M. tuberculosis* species (Parvez *et al.*, 2016). High conservation at protein level and presence of these families only in *M. tuberculosis* (Parvez *et al.*, 2016) species further strengthen that *M. tuberculosis* P450s can serve as drug targets and inhibitor developed against these families will be effective against TB.

Among these suspected TB-drug targets, there is one isoform that has shown unique characteristics. This is CYP51, which is involved in sterol 14 alpha-demethylation (Waterman and Lepesheva, 2005). CYP51 is the only P450 in *M. tuberculosis* that has a well-defined structure with its conformation in the open; it is also well characterised. An important feature of CYP51 is that it can be targeted by azole and triazole drugs (Odds *et al.*, 2003). Studies on CYP51 have made it evident that *M. tuberculosis* P450s play a crucial role in the cell and could be used as novel drug targets against *M. tuberculosis*.

### 2.4.3. Functional analysis of *M. tuberculosis* P450s

Of the 20 *M. tuberculosis* P450s, only five have been structurally and functionally characterised to date. They include CYP51B1 (Bellamine *et al.*, 1999), CYP121A1 (McLean *et al.*, 2002), CYP124A1 (Johnston *et al.*, 2009), CYP125A1 (McLean *et al.*, 2009) and CYP142A1 (Driscoll *et al.*, 2010). Two P450s, CYP130 (Ouellet *et al.*, 2008) and CYP144 (Driscoll, 2011), have only been structurally characterized (Table 2.2). The ones that have not been characterised are called "orphan P450s" and include all 15 other P450s. Table 2.2 shows, which CYPs have had their crystal structure analysed, where these CYPs are positioned and which anti-tuberculosis azole binds best to them.

**Table 2.2. Information on *M. tuberculosis* P450s crystal structure.**

| Crystallised P450 | Position (resolution) | Crystallisation form | Reference |
|---|---|---|---|
| **CYP51B1** | 2.2- 2.1 A° | Ligand free, 4-phenylimidazole, fluconazole, and estriol bound | Podust *et al.,* 2001; Podust *et al.,* 2004 |
| **CYP121A1** | 1.06 A° | Ligand free, fluconazole bound, and cYY bound | McLean *et al.,* 2008 |
| **CYP130A1** | 1.4 A° <br><br> 3.0 A° | Ligand free and econazole bound | Ouellet *et al.,* 2008 |
| **CYP124** | 1.5 A° <br><br> 2.1 A° | Ligand free and phytanic acid bound | Johnston *et al.,* 2009 |
| **CYP125A1** | 1.4 A° | Ligands and ligands free | McLean *et al.,* 2009 |

## 2.5. Aim of the study

*M. tuberculosis* is a human lung pathogen and well known for its ability to cause TB, a disease that is responsible for over 1.5 million deaths each year (Frieden *et al.,* 2003). The WHO has declared the epidemic a global health emergency (WHO, 2011). South Africa is a nation with one of the highest rates of TB-associated deaths. Co-occurrence of TB and HIV/AIDS further fuels the death rates, mainly because of TB rather than HIV/AIDS (Gandhi *et al.,* 2006). Although this is the most advanced medicine era yet, TB remains a major threat

to human health. The currently available anti-TB drugs were developed over 40 years ago. Furthermore, the development of MDR and XDR and the recent occurrence of totally drug-resistant (TDR) *M. tuberculosis* strains (Shenoi *et al.*, 2009; Migliori *et al.*, 2012), together with the paucity of new drug targets currently being explored, suggest that new basic research is required to identify novel potential targets.

Genome sequencing of *M. tuberculosis* (Cole *et al.*, 1998) revealed the presence of 20 P450s in its genome. P450s are mixed function oxidoreductases well known for their role in essential cellular anabolic and catabolic processes. As expected, three P450s, CYP121A1, CYP125A1 and CYP128A1, were found to be essential for the survival of *M. tuberculosis* (Sassetti and Rubin, 2003; Sassetti *et al.*, 2003; McLean *et al.*, 2008). *In vitro M. tuberculosis* latency model studies, including a carbon starvation model and hypoxia model, showed up-regulation of three *M. tuberculosis* P450s, CYP128A1 and CYP135A1 and CYP123A1, suggesting their potential role during *M. tuberculosis* latency (Betts *et al.*, 2002; Rustad *et al.*, 2008). Based on meta-analysis of expression data, CYP123A1 is selected as the best drug candidate against the dormant phase of *M. tuberculosis* (Murphy and Brown, 2007). It is noteworthy that CYP121A1 and CYP141A1 are present only in TB-causing bacteria (Parvez *et al.*, 2016) suggesting their essential role. Studies showed that P450s are essential for growth of *M. tuberculosis* on cholesterol and in assimilation of host fatty acids (Griffin *et al.*, 2011; Lee *et al.*, 2013). Taken together, the above studies strongly suggest that *M. tuberculosis* P450s represent novel anti-TB drug targets (Hudson *et al.*, 2012).

Despite the greater importance of *M. tuberculosis* P450s as novel drug targets, only five *M. tuberculosis* P450s have been functionally characterized for their *in vivo* role (Hudson *et al.*, 2012). The major challenges in *M. tuberculosis* P450 research are (i) expression of *M. tuberculosis* P450s and (ii) finding the physiological substrate(s). For example, even

leading *M. tuberculosis* P450 research groups were unable to express *M. tuberculosis* P450 CYP128A1 (Ouellet *et al.,* 2010; Driscoll, 2011). Recently, our laboratory at the Central University of Technology (CUT) achieved a major breakthrough in expression of previously difficult-to-express *M. tuberculosis* P450 CYP128A1 (Prof K Syed – personal communication).

The main aim of this project is to come up with a strategy to clone 14 *M. tuberculosis,* P450s and synthesizing and cloning of 14 *M. tuberculosis* P450s into novel expression vector and generation of recombinant *E. coli* cells containing *M. tuberculosis* P450 genes. Results from this work will pave the way for characterization of orphan *M. tuberculosis* P450s and development of *M. tuberculosis* P450 based anti-TB inhibitors.

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## CHAPTER 3

## DESIGNING A STRATEGY FOR CONSTRUCTION OF

## *MYCOBACTERIUM TUBERCULOSIS* P450 EXPRESSION LIBRARY

### 3.1. Introduction

Genome sequencing of *M. tuberculosis* generated crucial information regarding the *M. tuberculosis* proteomes, emphasizing the role of P450s in the survival and pathogenicity of this organism. A total of 20 P450s were found in this organism (Cole *et al.,* 1998). The high number of P450s found in *M. tuberculosis* shows that these P450s possibly have great significance in *M. tuberculosis* physiology. Most *M. tuberculosis* P450s characterized to date were found to be taking part in cholesterol metabolism (McLean *et al.,* 2009; García-Fernández *et al.,* 2013). There are two important P450 (CYP125 and CYP124) genes that play a role in *M. tuberculosis* cholesterol metabolism, even though *M. tuberculosis* does not have a *de novo* pathway for sterol synthesis (Cole *et al.,* 1998; McLean *et al.,* 2009; Driscoll *et al.,* 2010b). Azole drugs are known to be antifungal drugs because of their high affinity to P450 proteins, making them good candidates for an anti-TB drugs (Munro *et al.,* 2003). Quite a number of *M. tuberculosis* P450s have been listed as drug targets, including CYP121, CYP125, CYP128, and CYP142 (McLean *et al.,* 2002; McLean *et al.,* 2007; Holsclaw *et al.,* 2008; Capyk *et al.,* 2009; Driscoll *et al.,* 2010a). Among these P450s, CYP121, CYP125 and CPY128 are the most promising anti-TB drug targets, as these P450s have been found to be critical for *M. tuberculosis* survival (Hudson *et al.,* 2012). To date, only 7 of the 20 *M. tuberculosis* P450s have been EXPRESSED. Among those 7 seven P450s, 5 have been functionally characterised. Also, structural characterisation has been performed with 6 *M. tuberculosis* P450s and crystal structures are available (Figure 3.1 and Table 3.1).
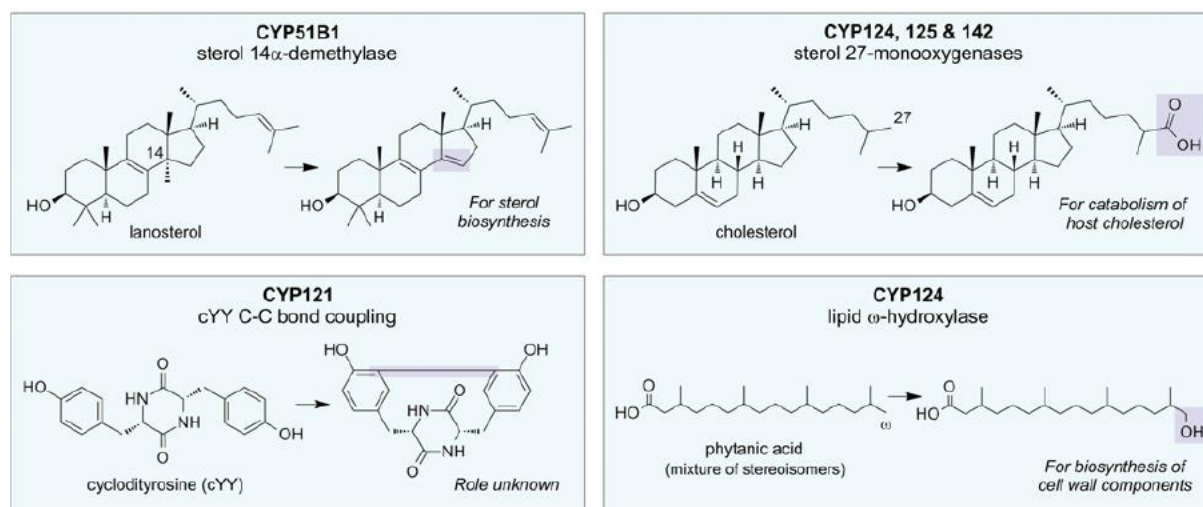
**Figure 3.1. Example of reactions catalysed by the *M. tuberculosis* P450s (taken from Hudson *et al.,* 2012)**. These include their putative cellular role in *M. tuberculosis* biochemistry. The functional group added/modified during catalysis is highlighted in purple.

**Table 3.1. Information on *M. tuberculosis* P450s characterized to date**.

| P450s | Expressed | Functionally Characterised | Crystal Structure Available | High affinity for Azoles | References |
|---|---|---|---|---|---|
| CYP51B1 | YES | YES | YES | YES | McLean *et al.,* 2002 |
| CYP121 | YES | YES | YES | YES | McLean *et al.,* 2008 |
| CYP124 | YES | YES | YES | NO | Johnston *et al.,* 2009 |
| CYP125 | YES | YES | YES | YES | McLean *et al.,* 2009 |
| CYP130 | YES | NO | YES | YES | Ouellet *et al.,* 2008 |
| CYP142 | YES | YES | YES | YES | Driscoll *et al.,* 2010b |
| CYP144 | YES | NO | NO | YES | Driscoll *et al.,* 2010a |

The *M. tuberculosis* P450s that have not been functionally characterized are called "orphan P450s". The major challenges in *M. tuberculosis* P450 research are (i) expression of *M. tuberculosis* P450s and (ii) physiological substrate(s) identification. In fact, even leading

*M. tuberculosis* P450 research groups have been unable to express *M. tuberculosis* P450 CYP128A1 (Ouellet *et al.*, 2010; Driscoll, 2011). Therefore, this chapter aims to come up with a strategy to clone 14 *M. tuberculosis* P450s in an expression vector. Considering the 7 *M. tuberculosis* P450s that have been successfully expressed in a heterologous host (Table 3.1), in this study, the remaining *M. tuberculosis* P450s were selected. The *M. tuberculosis* P450 CYP125A1 was selected as positive control.

## 3.2. Methodology

### 3.2.1. *M. tuberculosis* P450s and their IDs

A list of *M. tuberculosis* P450s used in this study and their gene IDs are shown in Table 3.2. The gene sequences were retrieved from the KEGG website (http://www.genome.jp/kegg/kegg1.html).

**Table 3.2. List of *M. tuberculosis* P450s and IDs used in this study**.

| CYP | CYP ID |
|---|---|
| CYP123A1 | Rv0766c |
| CYP125A1 | Rv3545c |
| CYP126A1 | Rv0778 |
| CYP128A1 | Rv2268c |
| CYP132A1 | Rv1394c |
| CYP135A1 | Rv0327c |
| CYP135B1 | Rv0568 |
| CYP136A1 | Rv3059 |
| CYP137A1 | Rv3685c |
| CYP138A1 | Rv0136 |
| CYP139A1 | Rv1666c |
| CYP140A1 | Rv1880c |
| CYP141A1 | Rv3121 |
| CYP143A1 | Rv1785c |

### 3.2.2. Information on expression vector

The expression vector (pINK-A) was a kind gift from Dr Naheed Kaderbhai, Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion, SY23 3DD, United Kingdom. The vector's multiple cloning site (MCS) was modified and used in this study. Since the work is going to be patented and commercial aspects are involved, details on the expression vector are not given.

### 3.2.3. Restriction enzyme analysis

Restriction enzyme analysis of genes and the expression vector was performed using freely available pDRAW DNA analysis software (http://www.acaclone.com/). Restriction enzymes and their sequences for creating a new multiple cloning site in the expression vector were downloaded from the New England Biolabs (NEB) website (https://www.neb.com/products/restriction-endonucleases).

### 3.2.4. Primer design

Primers for the cloning of *M. tuberculosis* P450s in the expression vector were designed *in silico* using IDT DNA OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer). Forward and reverse primer sequences were selected and after incorporating the appropriate restriction enzyme sequence, their melting temperature was measured using OligoAnalyzer. Primers were designed in such a way that both forward and reverse primers had matching melting temperatures.

## 3.3. Results and discussion

### 3.3.1. Modifying the multiple cloning site of the expression vector

The expression vector contained only a few restriction enzyme's recognition sites in its MCS. Therefore, the MCS was re-engineered by incorporating more recognition sites in order to clone *M. tuberculosis* P450s. The vector can also be used in future for cloning other genes. The expression vector sequence was analyzed using pDRAW and restriction enzymes that did not have recognition sites on the expression vector's sequence were noted. Based on the above criteria, a few of those restriction enzymes were selected and incorporated in the MCS of the expression vector. The restriction enzymes selected are listed in Table 3.3. The revised vector is named as pINK-d and its map and other features are shown in Figure 3.2.

**Table 3.3. List of selected restriction enzymes incorporated in the multiple cloning site of the expression vector.** The restriction enzyme and its recognition sequences are shown in the table.

| Restriction enzyme | Recognition Sequences (5' to 3') |
|---|---|
| AbsI | CC'TCGAGG |
| AflII | C'TTAAG |
| AgeI | A'CCGGT |
| AscI | GG'CGCGCC |
| AvrII | C'CTAGG |
| BglII | A'GATCT |
| BsiWI | C'GTACG |
| BspEI | T'CCGGA |
| BssHII | G'CGCGC |

| FseI | GGCCGG'CC |
|------|-----------|
| KasI | G'GCGCC |
| MfeI | C'AATTG |
| NcoI | C'CATGG |
| PluTI | GGCGC'C |
| SacI | GAGCT'C |
| SbfI | CCTGCA'GG |
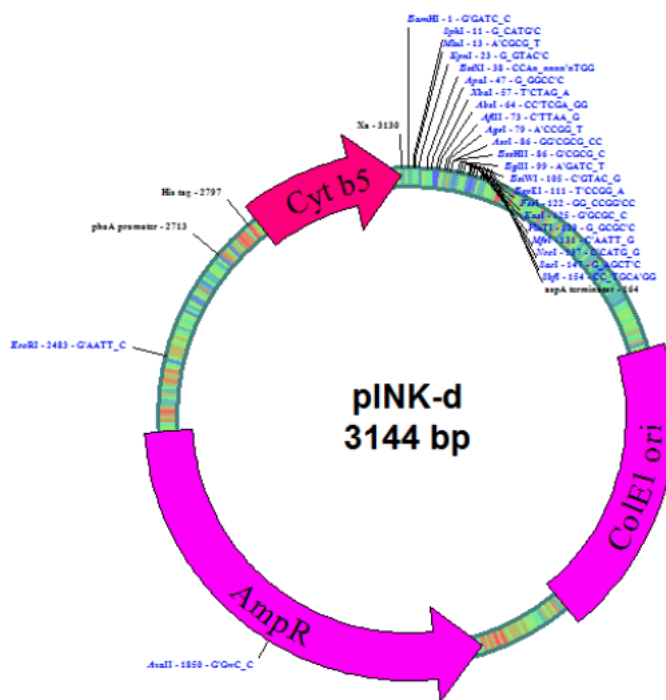


**Figure 3.2. Vector map of pINK-d.** Restriction sites present in the MCS are shown in blue font.

### 3.3.2. Strategy for cloning of *M. tuberculosis* P450s in expression vector

In order to design a strategy to clone 14 *M. tuberculosis* P450s in the expression vector, restriction enzyme profiling of the 14 *M. tuberculosis* P450s was carried out using pDRAW. Figure 3.3 shows the restriction profiling of the 14 *M. tuberculosis* P450s against pINK-d MCS enzymes. The restriction enzymes that do not cut the *M. tuberculosis* P450s and are part of the expression vector's MCS were selected for in-frame cloning of *M. tuberculosis* P450s (Figure 3.3).

| Enzymes | CYP123A1 | CYP125A1 | CYP126A1 | CYP128A1 | CYP132A1 | CYP135A1 | CYP135B1 | CYP136A1 | CYP137A1 | CYP138A1 | CYP139A1 | CYP140A1 | CYP141A1 | CYP143A1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BamHI | red | red | | | | | red | | | red | red | red | | red |
| BstXI | red | | | red | | red | | red | | red | red | | | |
| ApaI | red | | | red | | red | red | | | | | | red | red |
| EcoRV | | red | red | | | | | | | | | | | |
| KpnI | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow |
| MluI | | | | | | | | | red | | red | yellow | | |
| SphI | | | | | red | | | | | | | | red | |
| XbaI | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | yellow | red | yellow | yellow |

**Figure 3.3. Restriction enzyme profiling of 14 *M. tuberculosis* P450s**. For each *M. tuberculosis* P450, the red-filled boxes indicate that the corresponding restriction enzymes can cleave the gene. Two restriction enzymes with no cleaving ability were selected for each P450, for forward and reverse primers design, and are shown as yellow-filled boxes.

The selected restriction enzymes were made part of the forward and reverse primer of each *M. tuberculosis* P450, as shown in Table 3.4. Forward and reverse primers for each *M. tuberculosis* P450 were designed as described in the methodology. The primers were designed in such a way that they had almost same melting temperatures (Table 3.4.).

**Table 3.4.** **Primer sequences and restriction enzymes selected for cloning of 14**

*M. tuberculosis* **P450s**. The shaded regions represent the restriction enzyme recognition site.

The appropriate restriction enzyme in the forward primer (FP) and reverse primer (RP) is also

presented in the table.

| CYP          FP/RP | Primer sequences | Melting temp |
|---|---|---|
| CYP123A1-FP-KpnI: | TATATAGGTACCATGACCGTCCGCGTCGGTGACCCCG | (69.3) |
| CYP123A1-RP-XbaI: | GCCTTCTAGATTACCTGGCCTGCACGCTGATCGGC | (69.0) |
| | | |
| CYP125A1-FP-KpnI: | ATTACGGTACCATGGTGTCGTGGAATCACCAGTC | (64.8) |
| CYP125A1-RP-XbaI: | AGTAATGTCTCTAGATTAGTGAGCAACCGGGCATCTAC | (63.3) |
| | | |
| CYP126A1-FP-KpnI: | TATATTGGTACCATGACTACCGCCGCCGGGCTTTCGGG | (69.8) |
| CYP126A1-RP-XbaI: | ATATCTCTAGACTAGGTGCCGGATGCCGGTATGCCGGTTGC | (69.8) |
| | | |
| CYP128A1-FP-KpnI: | TAATGGTACCATGACCGCGACACAGTCCCCTCCCGAG | (69.7) |
| CYP128A1-RP-XbaI: | CGTCATCTAGATTATGGTGCGACGCGGGGGGTAACGGCGAC | (70.9) |
| | | |
| CYP132A1-FP-KpnI: | TAATTAGGTACCATGGCCACCGCCACCACCCAGC | (68.4) |
| CYP132A1-RP-XbaI: | GACCTATCTAGATTATCGCCTCCTCCCGATGACGTGTACTCC | (66.3) |
| | | |
| CYP135A1-FP-KpnI: | TATATGGTACCATGGCAAGCACGTTGACGACGGGCC | (68.5) |
| CYP135A1-RP-XbaI: | TTGGCTCTAGATTATGGCGGGTCACTGTCACCAAGCC | (68.0) |
| | | |
| CYP135B1-FP-KpnI: | ATGTTGGTACCATGAGCGGCACATCGTCGATGGGATTGCC | (69.8) |
| CYP135B1-RP-XbaI: | CTATGTCTAGATTACTGGCTGCCGACGGCTCTGGACGGC | (69.2) |
| | | |
| CYP136A1-FP-KpnI: | TATAGGGTACCATGGCGACGATCCACCCCCCGGCATAC | (70.5) |
| CYP136A1-RP-XbaI: | GATGCTCTAGACTACCTGGGACGCAGCACGATCGGCATCC | (70.0) |
| | | |
| CYP137A1-FP-KpnI: | ACGTAGGTACCATGGTGCTGAGGTCGCTTGCTAGCCCTG | (69.9) |
| CYP137A1-RP-XbaI: | GTACGTCTAGACTAACGGGCGGGGCGAAACCGCAC | (69.9) |
| | | |
| CYP138A1-FP-KpnI: | TATACGGTACCATGAGCGAAGTCGTCACCGCCGCACC | (69.9) |
| CYP138A1-RP-XbaI: | GTAGCTCTAGATTAGCGTCGGCGCATCACCACCCG | (68.4) |
| | | |
| CYP139A1-FP-KpnI: | TATAGGTACCATGGTGCGCTACCCGCTTGGCG | (68.3) |
| CYP139A1-RP-XbaI: | TGACATGCTCTAGATTACTGCGCTGGCGCTGACTTCCTG | (68.4) |
| | | |
| CYP140A1-FP-MluI: | GCGATCACGCGTATGGTGAAGGACAAGCTGCACTGGTTG | (69.3) |
| CYP140A1-RP-KpnI: | CGTTCGGTACCTCACGGGCTAACCATGGACCGTG | (69.0) |
| | | |
| CYP141A1-FP-KpnI: | GCTTGGTACCATGACAAGCACCTCGATTCCGACGTTCCC | (68.9) |
| CYP141A1-RP-XbaI: | GCGCTTCTAGATCACGTCGGCCAGGTAACAAGGAGTTCC | (68.4) |
| | | |
| CYP143A1-FP-KpnI: | TATATAGGTACCATGACCACCCCCGGCGAGGACC | (67.9) |
| CYP143A1-RP-XbaI: | ATGCATCTAGATCAGCTCCAGCGTAGCGGCAAGTTC | (67.0) |

In theory, after gene synthesis with the designed forward and reverse primers, the synthesized products will be digested and cloned into the expression vector with the appropriate restriction enzymes. The vector maps with 14 cloned *M. tuberculosis* P450s were shown in Figure 3.4.

**Figure 3.4. Vector maps showing the cloning of 14 *M. tuberculosis* P450s in pINK-d.**
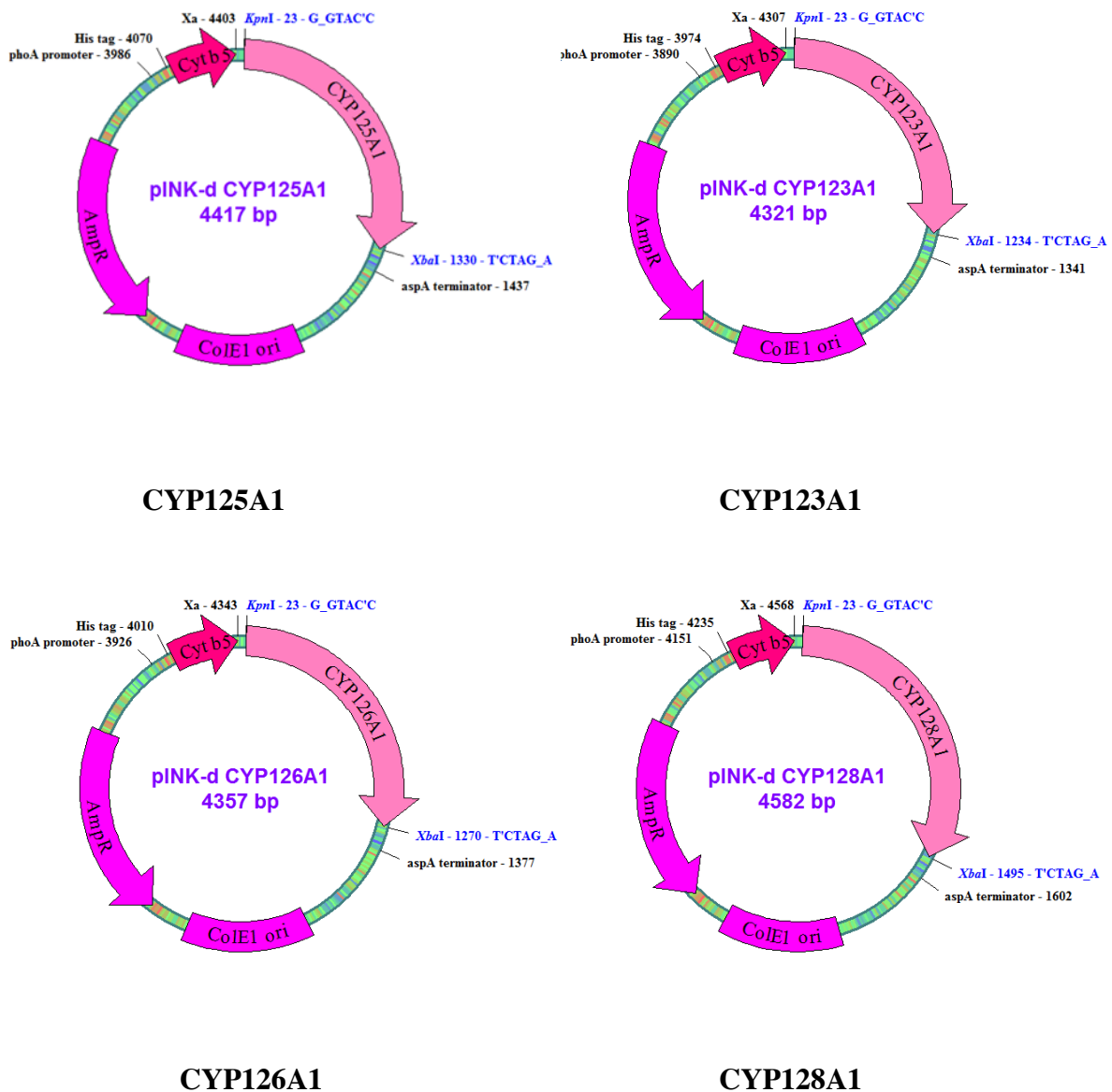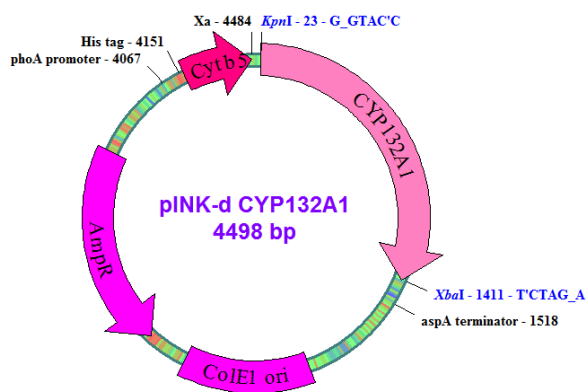


CYP125A1

CYP123A1

CYP126A1

CYP128A1

CYP132A1
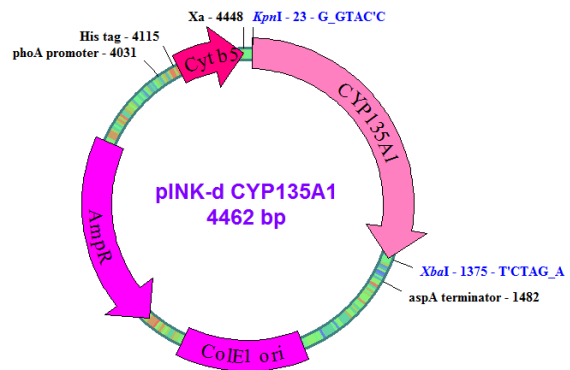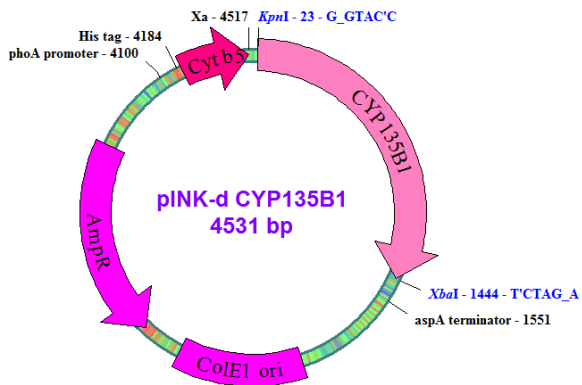


CYP135A1



CYP135B1
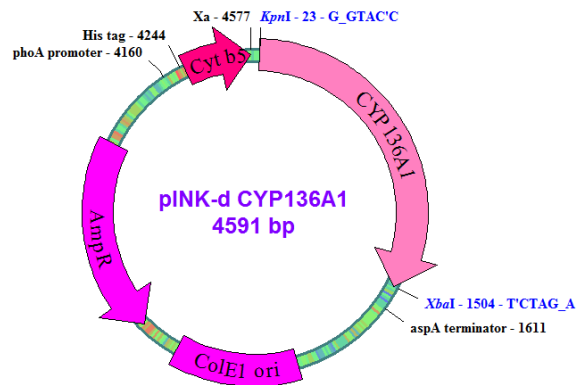


CYP136A1
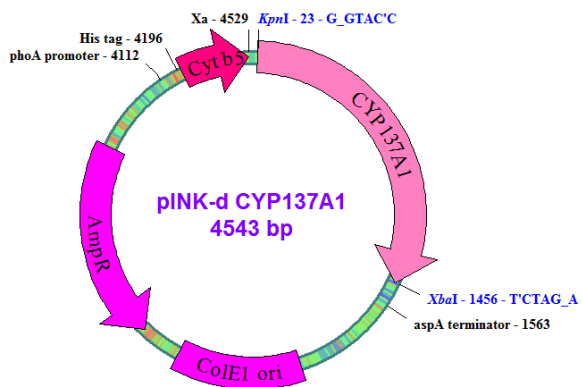


CYP137A1
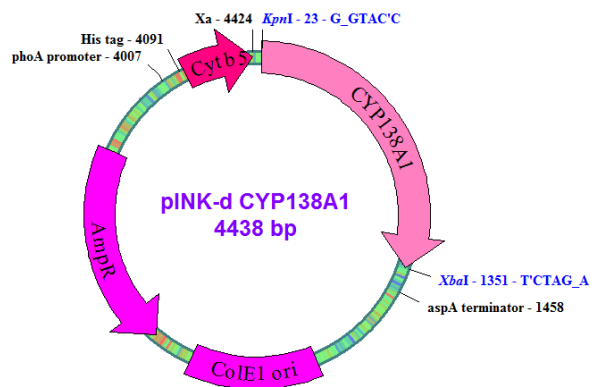


CYP138A1
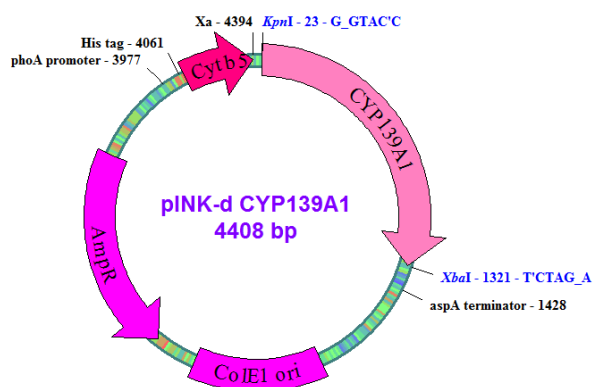
**CYP139A1**
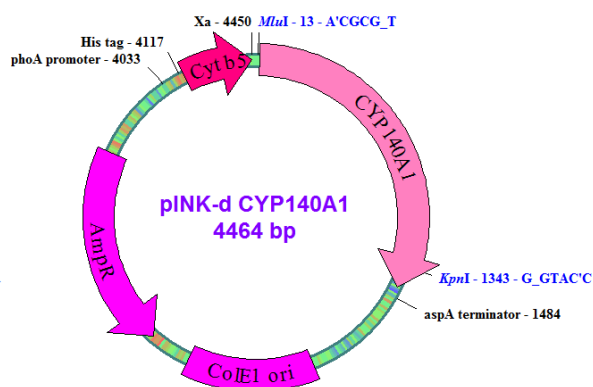


**CYP140A1**
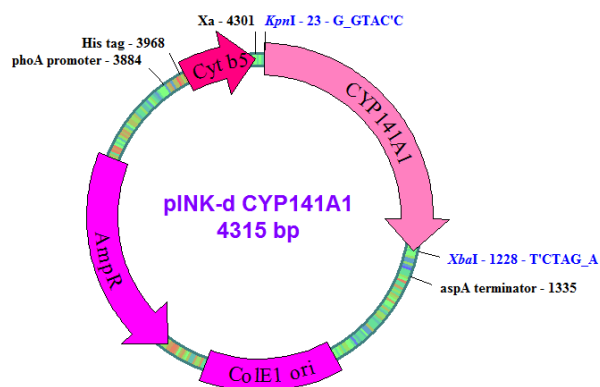


**CYP141A1**
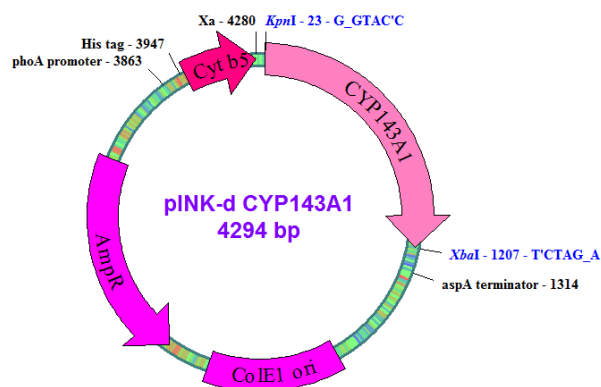


**CYP143A1**

**3.4. Conclusion**

In conclusion, the multiple cloning site of the pINK-A vector was successfully modified, by adding more suitable restriction enzymes and the revised vector named as pINK-d. A total of 16 restriction enzymes were added in such a way that they did not cause any shift or change to the reading frame of the vector. The new vector can be used in the future for the cloning of other P450s. Forward and reverse primers were also carefully designed to contain appropriate restriction enzymes site. The developed strategy will be used to synthesize vector and genes and clone the genes in the expression vector.

61

## 3.5. References

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# CHAPTER 4

# CONSTRUCTION OF WORLD'S FIRST *MYCOBACTERIUM TUBERCULOSIS* P450 LIBRARY IN *ESCHERICHIA COLI*

## 4.1. Introduction

A lot of research has been going on with regards to *M*. *tuberculosis* P450s (McLean *et al*., 2002; Sassetti *et al*., 2003; McLean *et al*., 2008; Ouellet *et al*., 2008; Johnston *et al*., 2009). However, researchers have not been successful in expressing *M*. *tuberculosis* P450s or finding a suitable substrate(s) for the orphan P450s (Ouellet *et al*., 2010; Driscoll, 2011). Hence, in the previous chapter, a strategy was developed to clone 14 *M*. *tuberculosis* P450s using pINK-d.

DNA synthesis using *de novo* process has many advantages over the traditional techniques (Table 4.1) (GenScript, 2014). The advantage of using gene synthesis is that the researcher only specifies the sequence of interest and the gene is directly custom-built without any problems. This process is cost effective and saves a lot of time. Even tasks that look easy, like gene isolation using PCR cloning, can be tedious and error-prone, depending on the sequence and its context in the source material. Most cDNAs that were predicted by genomics have proved difficult to clone but easy to synthesise. Hence, most people prefer gene synthesis instead of traditional molecular cloning. Therefore, gene synthesis forms the foundation of the new field of synthetic biology. Gene synthesis has become a solution to the limitation of laborious traditional molecular cloning techniques. The following Table 4.1 illustrates the applications of synthetic DNA.

**Table 4.1. Examples of synthetic DNA and their applications** (taken from GenScript, 2014).

| Synthetic DNA | Application |
|---|---|
| cDNA/ORFs | Over-expression or heterologous |
| Customized coding sequence | Expressing fusion protein; high-level protein expression from codon- optimized sequences for purification for enzymatic or structural studies |
| Promoter-reporter construct | Monitoring gene expression downstream of manipulations to transcription factors, signalling cascades. |
| Genomic DNA | Creating synthetic genes or genomes; studying gene structure, regulation, and evolution. |
| Mutant sequences | Confirming function ; protein engineering |
| RNAi constructs (shRNA, miRNA, siRNA) | Regulating gene expression; intercellular communication. |
| Extracellular DNA | Biofilm formation, intercellular signalling as in cancer metastasis. |

Gene synthesis started 67 years ago with Sir Alexander Todd when the first DNA molecule was synthesized by joining two thymidine nucleotides using a phosphate bond (GenScript, 2014). Later, in the year 1970 Gobind Khorana and his group started synthesizing the tryrosine specific bacterial tRNA and it took them 3years before they could finish the synthesis and get the protein to work (Entelechon, 2012). Figure 4.1 illustrates historical milestones in gene synthesis. Due to the improving standards of research and inventions the work that was done in 3-5 years is now completed in hours. The same year, after the

announcement of the Khorana success, Genetech, a genetics laboratory, invented an

automated method for synthesizing whole genes (Entelechon, 2012). However, it took them a

long time before they could commercialise it.  In the 90s long genes were also synthesized,

showing the improvements in gene synthesis (Figure 4.1).
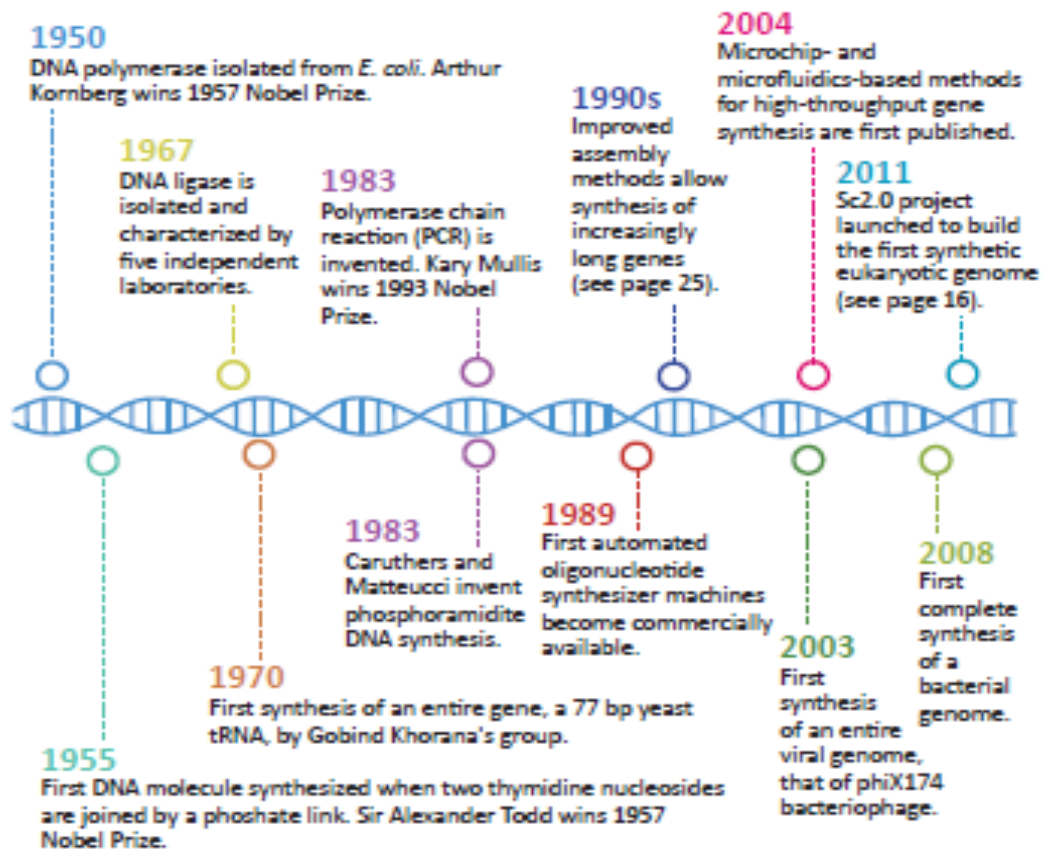


**Figure 4.1. History of gene synthesis technology** (taken from GenScript, 2014).

Every scientific method has a protocol and the same goes for gene synthesis. Figure 4.2 below shows the following steps that are followed during this process.
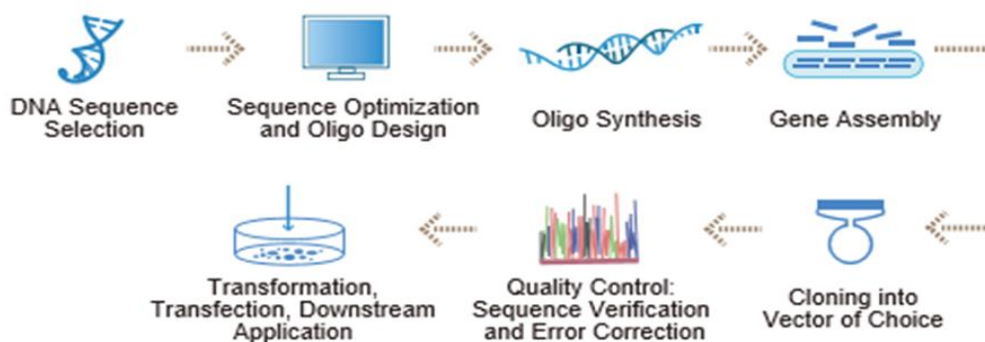


**Figure 4.2. Sequential steps in gene synthesis** (taken from GenScript, 2014).

Our laboratory does not have permission to culture *M. tuberculosis* H37Rv strain and also the problems occurred to obtain genomic DNA from different sources. Hence, in this study, *de novo* synthesis of 14 *M. tuberculosis* P450 genes and further, cloning into the modified expression vector is carried out. The work in this chapter is aimed to clone 14 *M. tuberculosis* P450s in modified vector, transform the created vectors into *E. coli*, isolate vectors from recombinant *E. coli* and then confirm the gene insert by restriction digestion.

**4.2. Materials and Methods**

**4.2.1. Strains, plasmids, chemicals and kits**

*E. coli* DH5α strain was used in this study. *E. coli* cells were cultured on Luria-Bertani (LB) broth and LB-agar (LB broth supplemented with 10 g/L agar). For selection of recombinant *E. coli* cells LB was supplied with antibiotic ampicillin. LB-antibiotic plates were prepared by adding ampicillin at 100 μg/ml final concentration. Ampicillin stock solution was prepared by dissolving 100 mg of ampicillin (Catalog No. A6140, Sigma-Aldrich, USA) in 1 ml of DNase and RNase free water (Catalog No. L3152, Sigma-Aldrich, USA). The

ampicillin stock solution was stored at -20$^{o}$C. The pINK-d expression vector, as mentioned in the previous chapter, was used for cloning the 14 *M. tuberculosis* P450s. All chemicals used were high grade quality and were purchased from Sigma-Aldrich and Merck. Plasmid isolation kit was purchased from Qiagen, USA.

### 4.2.2. Synthesis and cloning of 14 *M. tuberculosis* P450s

In the previous chapter a strategy was designed to clone 14 *M. tuberculosis* P450s. The *M. tuberculosis* P450 cDNA sequences along with modified vector sequence and cloning strategy were submitted to GenScript (GenScript USA Inc, USA). GenScript synthesized all 14 *M. tuberculosis* P450s and cloned into the modified vector and the constructs were received.

### 4.2.3. Preparation of competent cells and transformation

Recombinant plasmids were propagated using chemically competent *E. coli* DH5α, according to the methods described by Inoue *et al.,* (1990). To prepare competent cells, LB broth was inoculated with a glycerol stock of the relevant cells, and incubated at 37°C for approximately 10 hours. This pre-culture was then used to inoculate fresh 250 ml of SOB medium (20 g/L tryptone, 5 g/L yeast extract, 0.584 g/L NaCl, 0.186 g/L KCl, 2.034 g/L MgCl2, 2.464 g/L MgSO4), which was then incubated overnight at 18-20°C until OD$_{600}$ ~0.55. The culture was then incubated on ice for 10 minutes, followed by centrifugation at 2500 x g for 10 minutes at 4°C. The cell pellet was resuspended on ice in 80 ml of ice-cold TB buffer (10 mM HEPES, pH 6.7; 15mM CaCl2; 250 mM KCl; 55 mM MnCl2), and incubated on ice for 10 minutes. The resuspension was then centrifuged at 2500 g for 10 minutes at 4°C, followed by resuspension in 20 ml ice-cold TB buffer supplemented with DMSO (7% v/v). This was incubated on ice for 10 minutes, before aliquoting 100 µl per 1.5 ml microcentrifuge tube.

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69

To transform the cells, 1 µl plasmid solution containing each of the *M. tuberculosis* P450 genes cloned in expression vector (obtained from GenScript), was added to the 100 µl competent cells aliquot, and incubated on ice for 20 minutes. Cells were heat-shocked at 42°C for 1 minute, followed by cold-shock on ice for 2 minutes. Thereafter, 250 µl SOC medium (SOB medium supplemented with 20 mM glucose) was added to the cells, which were then incubated at 37°C for one hour. The solution was then centrifuged at 2500 g for 5 minutes, after which 150 µl supernatant was removed and the cells re-suspended in the remaining 100 µl. The re-suspended cells were streaked on LB agar plates supplemented with 100 µg/ml ampicillin and incubated at 37°C for 16 hours.

### 4.2.4. Plasmid isolation and purification

Plasmid isolation and purification of the recombinant cells were carried out using QIAprep Spin Miniprep Kit (Catalog No. 27104, Qiagen, Germany) following the manufacturer's protocol. Plasmid DNA concentration was carried out using SimpliNano microvolume spectrophotometer (Catalog No. GE29-0617-13, Sigma-Aldrich, USA).

### 4.2.5. Restriction enzyme analysis of plasmids

The above isolated plasmids from recombinant *E. coli* cells were subjected to restriction enzyme digestion to check the presence of the inserts and the correct size of the cloned *M. tuberculosis* P450 cDNAs. All restriction enzymes used in this study were purchased from New England Biolabs, South Africa. Digested DNA fragments were analysed on 1% agarose gels. Visualization of DNA fragments were carried out using SYBR® Sae DNA gel stain (Catalog No. S33102, Thermo Fisher Scientific, USA). The agarose gels were photographed using Gel Doc<sup>TM</sup> EZ System (Bio-Rad, South Africa).

## 4.3. Results and discussion

### 4.3.1. GenScript synthesis of 14 *M. tuberculosis* P450 cDNAs and cloning into expression vector

All 14 *M. tuberculosis* P450 cDNAs along with the expression vector were synthesized by GenScript, USA. The constructs carrying individual *M. tuberculosis* P450 cDNAs and empty expression vector were received in a microtiter plate. The constructs and the expression vector were resuspended in 100 µl of Tris-Hcl buffer (pH: 8.0) and 1 µl of this solution was used for transformation.

### 4.3.2. Generation of recombinant *E. coli* cells containing *M. tuberculosis* P450 genes

The 14 constructs containing the individual *M. tuberculosis* P450 cDNAs were transformed into *E. coli* DH5α using the transformation method described in the "Materials and methods" section (Inoue *et al*., 1990). The recombinant cells were selected on LB-agar ampicillin plates. The figure below indicates the growth of recombinant cells on ampicillin containing plates. This indicates that the constructs and expression vector were successfully transformed in to *E. coli*.
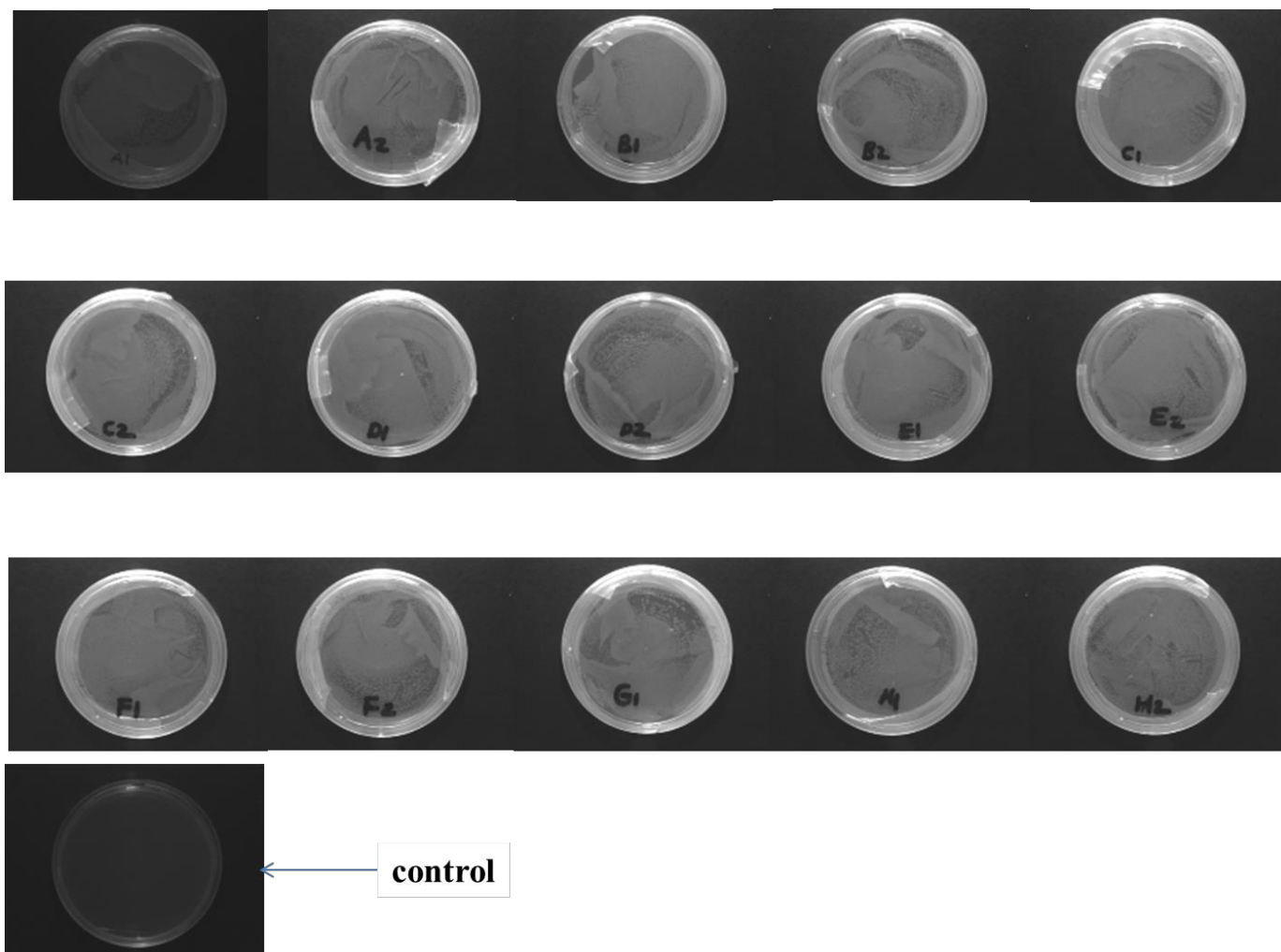
**Figure 4.3. Selection of transformed *E. coli* DH5α on LB medium plates containing ampicillin antibiotic.** A1: pINK-d_CYP123A1; A2: pINK-d_CYP137A1; B1: pINK-d_CYP125A1; B2: pINK-d_CYP138A1; C1: pINK-d_CYP126A1; C2: pINK-d_CYP139A1; D1: pINK-d_CYP128A1; D2: pINK-d_CYP140A1; E1: pINK-d_CYP132A1; E2: pINK-d_CYP141A1; F1: pINK-d_CYP135A1; F2: pINK-d_CYP143A1; G1: pINK-d_CYP135B1; H1: pINK-d_CYP136A1; H2: pINK-d vector and the control 1 plate: plate speared with only *E. coli* cells. No growth on control plate indicates no contamination during transformation.

From the above plates, a single colony was picked and plated on a separate LB-agar plate containing ampicillin. Figure 4.4 below shows the propagation of selected recombinant cells.
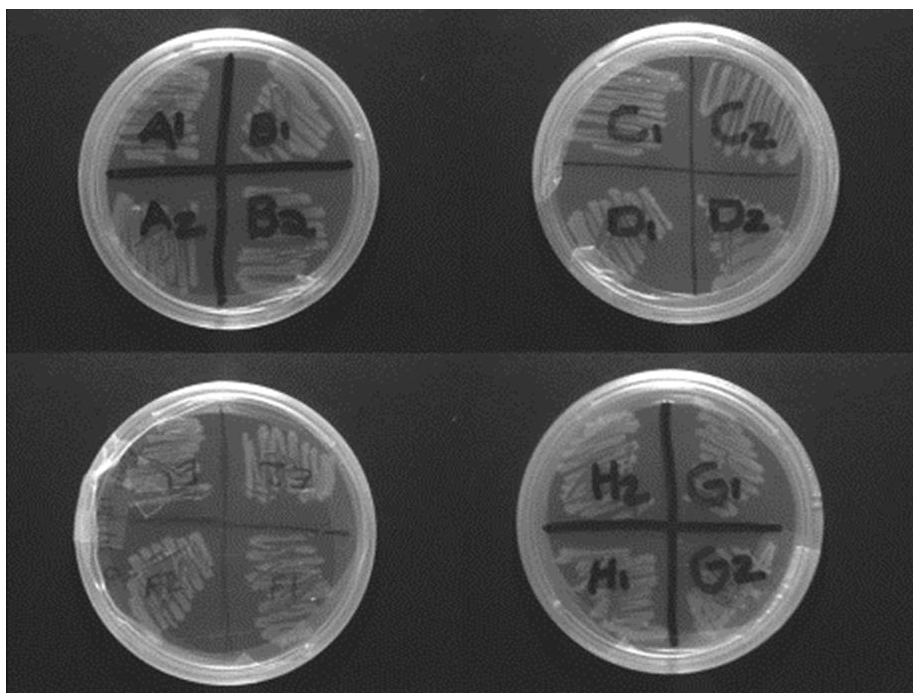
**Figure 4.4. Propagation of selected recombinant *E. coli* cells containing *M. tuberculosis* P450s.** The labels on plates follows *E. coli* cells containing respective gene: A1: pINK-d_CYP123A1; A2: pINK-d_CYP137A1; B1: pINK-d_CYP125A1; B2: pINK-d_CYP138A1; C1: pINK-d_CYP126A1; C2: pINK-d_CYP139A1; D1: pINK-d_CYP128A1; D2: pINK-d_CYP140A1; E1: pINK-d_CYP132A1; E2: pINK-d_CYP141A1; F1: pINK-d_CYP135A1; F2: pINK-d_CYP143A1; G1: pINK-d_CYP135B1; H1: pINK-d_CYP136A1; H2: pINK-d vector.

### 4.3.3. Plasmid isolation and confirming the presence of *M. tuberculosis* P450 cDNA

The above selected recombinant *E. coli* cells carrying individual *M. tuberculosis* P450 cDNAs and expression vector were inoculated into 10 ml of LB broth and incubated at 37$^{\circ}$C and 150 rpm for overnight. The overnight bacterial culture was used for isolation of plasmids. The plasmid DNAs were extracted using QIAprep Spin Miniprep Kit and the concentration of the plasmid DNA was measured (Table 4.2) before being subjected to restriction enzyme digestion analysis with enzymes specified in Table 4.2.

**Table 4.2. Recombinant plasmid DNA concentration and enzymes used for releasing the inserted cDNA.**

| Name of the recombinant plasmids | Yield ($\mu$l/ ml) | Enzymes |
|---|---|---|
| pINK-d_CYP123A1 | 237.6 | KpnI and XbaI |
| pINK-d_CYP125A1 | 275.5 | KpnI and XbaI |
| pINK-d_CYP126A1 | 281.2 | KpnI and XbaI |
| pINK-d_CYP128A1 | 261.2 | KpnI and XbaI |
| pINK-d_CYP132A1 | 303.3 | KpnI and XbaI |
| pINK-d_CYP135A1 | 354.2 | KpnI and XbaI |
| pINK-d_CYP135B1 | 255.4 | KpnI and XbaI |
| pINK-d_CYP136A1 | 266.1 | KpnI and XbaI |
| pINK-d_CYP137A1 | 301.2 | KpnI and XbaI |
| pINK-d_CYP138A1 | 270.8 | KpnI and XbaI |
| pINK-d_CYP139A1 | 241.6 | KpnI and XbaI |
| pINK-d_CYP140A1 | 272.6 | MluI and KpnI |
| pINK-d_CYP141A1 | 258.0 | KpnI and XbaI |
| pINK-d_CYP143A1 | 255.6 | KpnI and XbaI |
| pINK-d | 287.4 | EcoRI and HindIII |

After digestion of plasmid DNAs, the digested products were run on a 1% agarose gel. The figure below (Figure 4.5) shows the results of the gel electrophoresis, which indicate that all 14 *M. tuberculosis* P450 cDNAs were cloned using the suggested restriction enzymes and thus correct size of insert was released upon digestion.
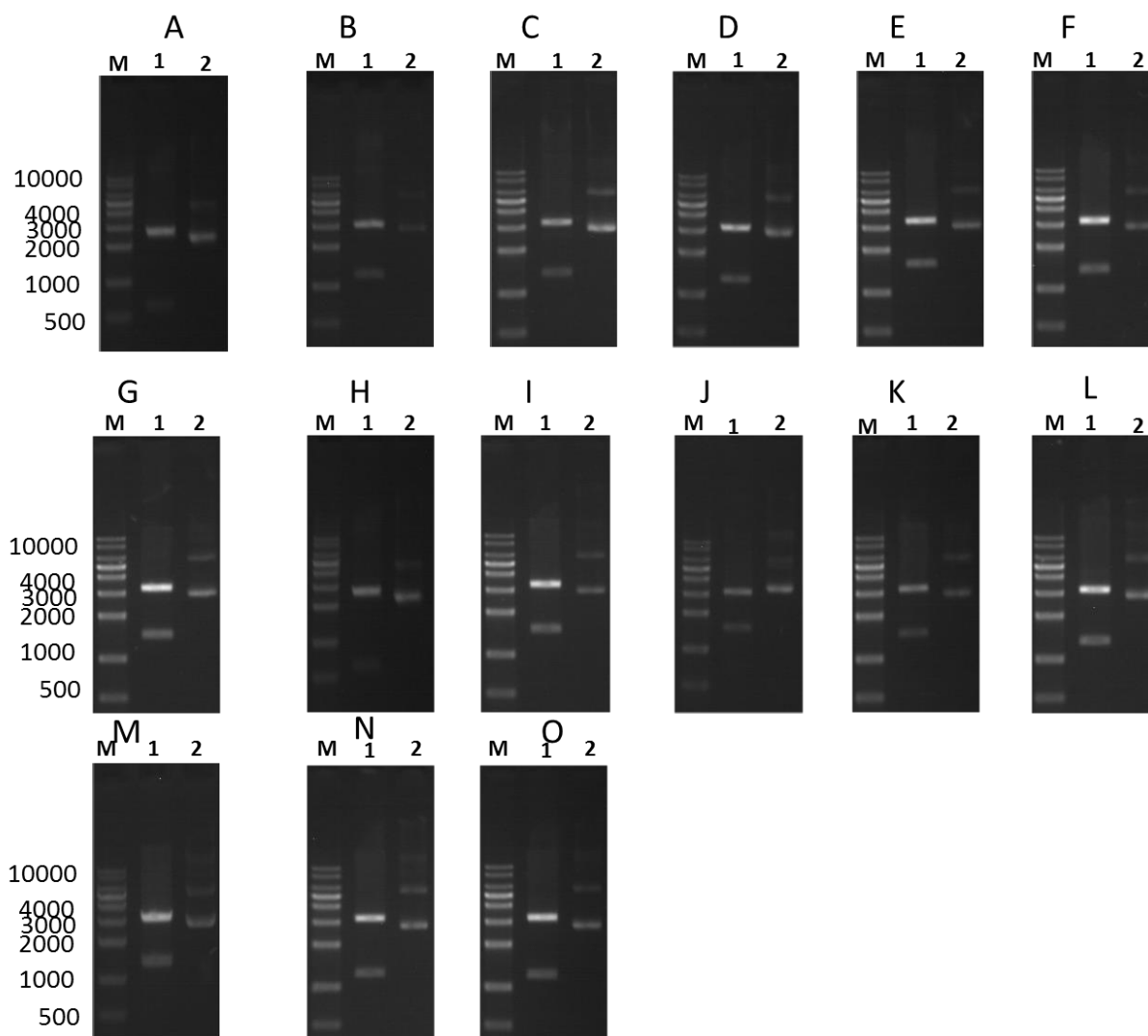
**Figure 4.5. Restriction enzyme digestion analysis of recombinant *M. tuberculosis* P450 cDNA plasmids.** 300 ng of each plasmid was digested in a water bath at 37°C for 40 min, and then later run on a 1% agarose gel. Panels A to O indicate: A: pINK-d, B: pINK-d_CYP123A1, C: pINK-d_CYP125A1, D: pINK-d_CYP126A1, E: pINK-d_CYP128A1, F: pINK-d_CYP138A1, G: pINK-d_CYP132A1, H: pINK-d_CYP135A1, I: pINK-d_CYP135B1, J: pINK-d_CYP136A1, K: pINK-d_CYP137A1, L: pINK-d_CYP139A1, M: pINK-d_CYP140A1, N: pINK-d_CYP141A1 and O: pINK-d_CYP143A1. In all panels Lane M represents molecular weight markers (1 KB DNA ladder) and Lanes 1 and 2 represent

digested and un-digested plasmid, respectively. The respective enzymes used for digestion of plasmids were shown in Table 4.1.

## 4.4. Conclusion

In the current study, as part of this chapter, all 14 *M. tuberculosis* P450 cDNAs and the modified expression plasmid were successfully synthesized by GenScript. All synthesized 14 *M. tuberculosis* P450s were further cloned into expression vector. The constructs were transformed into *E. coli* cells and recombinant cells were selected. Plasmids were isolated and the presence of correct size inserts was verified by restriction enzyme digestion analysis. All recombinant *E. coli* cells carrying individual *M. tuberculosis* P450s and expression vector were stored at -80$^{\mathrm{o}}$C for further expression analysis of *M. tuberculosis* P450s.

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## CHAPTER 5

## CONCLUSION AND FUTURE PESPECTIVES

Tuberculosis (TB) is a global epidemic with highest mortality in the world. TB is responsible for over 1.5 million deaths each year. The World Health Organization has declared the epidemic a global health emergency. South Africa is one of the countries with the highest rate of TB-associated deaths. Co-occurrence of TB and HIV/AIDS further fuels the death rates, mainly because of TB rather than HIV/AIDS. Despite living in the most advanced medicine era, TB remains a major threat to human health. Development of drug-resistant strains suggests that new basic research is required to delineate novel potential targets. Genome sequencing of *Mycobacterium tuberculosis*, the causative agent of TB, revealed the presence of 20 cytochrome P450 monooxygenases (P450s) in its genome. P450s are mixed function oxidoreductases well known for their role in essential cellular anabolic and catabolic processes.

Researchers across the world are trying to find novel drug targets to develop new drugs. In this direction, research has shown that P450s of *M. tuberculosis* can serve as a novel drug target. However, the progress in utilizing *M. tuberculosis* P450s as drug targets is hampered by failure in expression and also not finding substrate(s).

In this direction, this study is the first of its kind in the designing of a novel expression vector and cloning of 14 *M. tuberculosis* P450s. All the constructs were successfully transformed into *E. coli* and the presence of the inserts were verified. The recombinant *E. coli* cells were stored for future expression analysis of *M. tuberculosis* P450s. This work will provide a platform for the expression and characterisation of the entire remaining orphan P450s.

# RESEARCH OUTPUTS

**Research articles**

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## Molecular evolutionary dynamics of cytochrome P450 monooxygenases across kingdoms: Special focus on mycobacterial P450s

Mohammad Parvez[1], Lehlohonolo Benedict Qhanya[1], Ntsane Trevor Mthakathi[1], Ipeleng Kopano Rosinah Kgosiemang[1], Hans Denis Bamal[1], Nataraj Sekhar Pagadala[2], Ting Xie[3], Haoran Yang[4], Hengye Chen[4], Chrispian William Theron[5], Richie Monyaki[1], Seiso Caiphus Raselemane[1], Vuyani Salewe[1], Bogadi Lorato Mongale[1], Retshedisitswe Godfrey Matowane[1], Sara Mohamed Hasaan Abdalla[1], Wool Isaac Booi[1], Mari van Wyk[1], Dedré Olivier[1], Charlotte E. Boucher[5], David R. Nelson[6], Jack A. Tuszynski[7], Jonathan Michael Blackburn[8], Jae-Hyuk Yu[9], Samson Sitheni Mashele[1], Wanping Chen[4] & Khajamohiddin Syed[1]

# SCIENTIFIC REP⚙RTS

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## Diversity and evolution of cytochrome P450 monooxygenases in Oomycetes

Mopeli Marshal Sello[1], Norventia Jafta[1], David R Nelson[2], Wanping Chen[3], Jae-Hyuk Yu[4], Mohammad Parvez[1], Ipeleng Kopano Rosinah Kgosiemang[1], Richie Monyaki[1], Seiso Caiphus Raselemane[1], Lehlohonolo Benedict Qhanya[1], Ntsane Trevor Mthakathi[1], Samson Sitheni Mashele[1] & Khajamohiddin Syed[1]

# Cytochrome P450 monooxygenase analysis in free-living and symbiotic microalgae *Coccomyxa* sp. C-169 and *Chlorella* sp. NC64A

Ntsane Trevor Mthakathi[1], Ipeleng Kopano Rosinah Kgosiemang[1], Wanping Chen[2], Molikeng Eric Mohlatsane[1], Thebeyapelo Jacob Mojahi[1], Jae-Hyuk Yu[3], Samson Sitheni Mashele[1] and Khajamohiddin Syed[1,*]

[1]*Unit for Drug Discovery Research, Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9300, Free State, South Africa*
[2]*College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China*
[3]*Department of Bacteriology, University of Wisconsin-Madison, 3155 MSB, 1550 Linden Drive, Madison, WI 53706, USA*

## Conference Attendance



13th International Symposium on Cytochrome P450 Biodiversity and Biotechnology

22-26 July, 2016
Vancouver, B.C., Canada
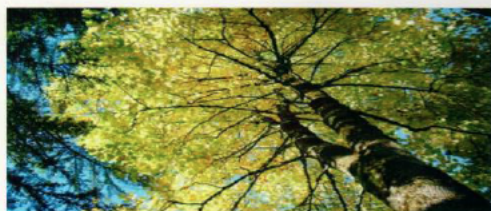
Program and Abstracts

P 76-S2

### Construction and validation of *Mycobacterium tuberculosis* P450 monooxygenase expression library

Kgosiemang, Ipeleng Kopano Rosinah; Mashele, Samson Sitheni; Syed, Khajamohiddin

Unit for Drug Discovery Research, Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, South Africa. Email: kopanokgosiemang@ymail.com

The actinomycete *Mycobacterium tuberculosis* (Mtb) causes Tuberculosis, a chronic lung disease, in humans and continues to be one of the greatest threats to mankind. Development of multidrug-resistant, extensively drug-resistance and recent occurrence of totally drug-resistant *Mtb* strains together with the paucity of new drug targets currently being explored, suggests that new basic research is required to delineate novel potential targets. Genome sequencing of *Mtb* revealed the presence of 20 cytochrome P450 monooxygenases (P450s) in its genome. P450s are mixed function oxidoreductases well known for their role in essential cellular anabolic and catabolic processes. Large number of studies showed that *Mtb* P450s can be used as novel drug target. Despite the greater importance only five *Mtb* P450s among 20 P450s that have been functionally characterized. The major challenges in *Mtb* P450 research is the expression of its P450s and functional identification. In this study, 20 *Mtb* P450 genes were cloned in a novel expression vector and twenty recombinant *E. coli* cells containing a single *Mtb* P450s were created. Expression of *Mtb* P450s was analysed using P450 spectrum and western blotting. This is the first report on construction of world's first *Mtb* P450 expression library.

49

**DMP 14**

**Restriction enzyme profiling of *Mycobacterium tuberculosis* cytochrome P450 monooxygenases and its redox partners**

I.K.R. Kgosiemang, S.S. Mashele and K. Syed

Unit for Drug Discovery Research, Department of Health Sciences, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein 9301, South Africa

The actinomycete *Mycobacterium tuberculosis* (*Mtb*) causes Tuberculosis (TB), a chronic lung disease, in humans and continues to be one of the greatest threats to mankind. TB, a pre historic disease, still remains a leading cause of death worldwide, despite global efforts in disease-control programs during the past 20 years. It is estimated that one-third of the human population are latently infected by *Mtb* and 9 million incidences of active TB occur each year. Development of multidrug-resistant, extensively drug-resistance and recent occurrence of totally drug-resistant *Mtb* strains together with the paucity of new drug targets currently being explored, suggests that new basic research is required to delineate novel potential targets. Genome sequencing of *Mtb* revealed the presence of 20 cytochrome P450 monooxygenases (P450s) in its genome. P450s are mixed function oxidoreductases well known for their role in essential cellular anabolic and catabolic processes. Large number of studies showed that *Mtb* P450s can be used as novel drug target. Despite the greater importance only five *Mtb* P450s among 20 P450s that have been functionally characterized. The major challenges in *Mtb* P450 research is the expression of its P450s. Therefore, this study aims to conduct restriction enzyme profiling for future construction and validation of microtiter plate-based *Mtb* P450 expression library in *E. coli*. In this direction, restriction enzyme analysis of 20 *Mtb* P450s and its redox partners (*FprA* and *FdX*) has been carried out. Different databases and programs such as New England BioLabs (https://www.neb.com/products/restriction-endonucleases) and pDRAW (http://www.acaclone.com/) has been utilized for identifying restriction enzymes that cut and uncut the 20 *Mtb* P450s and redox partners. Unique restriction enzyme that does not cut either *Mtb* P450s or redox partners was selected for cloning of these genes into *E. coli* expression vector.

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# CUT's groundbreaking discovery

New discovery may help fight aquatic animal infection

The New Age (Free State) · 17 Jun 2015 · News

leading: Research team leader Dr Khajamohiddin Syed and faculty of environmental science acting dean professor Sam Mashele.

pioneers: The CUT science students who helped discover the drug that may help fight aquatic animal infection.

Acting dean of the faculty Prof Sam Mashele said some of the deadliest pathogens in the world were found in the fish farming industry.

He said the discovery was made by researchers from the unit for drug discovery in the institution.

"For many years researchers across the world have been trying to understand these micro-organisms in order to control the disease and develop novel drugs against these pathogens, and CUT researchers are leading the way in finding solutions that will bring an end to this socio-economic challenge facing aquatic farming," Mashele said.

He said microorganisms were widely known in the scientific world and they continued to wreak havoc on the aqua farming sector worldwide.

"They are considered the deadliest of pathogens, causing diminished production of aquatic food," Mashele said.

The team of researchers who discovered the drug were led by Dr Khajamohiddin Syed.

"We collaborated with highly acclaimed international scientists such as professors David Nelson from the University of Tennessee in the US, aeHyuk Yu from the University of Wisconsin-Madison and Dr Wanping Chen from Huazhong Agricultural University in China," Mashele said.

"The university researchers are investigating solutions that would sustain the aquatic resources while helping to increase high production levels of aqua farming for commercial purposes, food security and poverty alleviation."

He said their work highlighted the important role aqua farming plays in promoting healthy living and in fighting poverty and hunger.

Syed said aqua farming was a big industry across the world and it involved the farming of fish, shrimps, prawns, squid and octopus and is considered by the UN as an important sector that can provide a livelihood for more than 60 million people in Africa and Asia.

"Consumption of these animals remains a vital source of protein and essential nutrients, especially for developing countries, where they constitute almost half of the total value of their traded communities," Syed said.

"The results of this study have been accepted for publication in the Nature Publication Group journal Scientific Reports, a prestigious multidisciplinary scientific international journal with an impact factor of 5.1."

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Maandag 16 Junie 2015   Volksblad

# Navorsers by SUT maak deurbraak

Zaydene Davids

'n SPAN navorsers aan die Sentrale Universiteit vir Tegnologie (SUT) het 'n wegspringpunt vir 'n middel ontdek wat groot potensiaal toon om siektes, wat deur parasiete veroorsaak word, in seediere te bestry.

Navorsers van oor die wêreld soek het jare lank probeer om die swamagtige mikro-organismes te verstaan ten einde die siekte, wat onder meer in visse, garnale, inkvisse en seekatte voorkom, te beheer en 'n middel daarteen te ontwikkel.

Maar meer as 50 jaar lank was dit tevergeefs – totdat die navorsers aan die SUT en wêreldkenners se wetenskaplike koppe bymekaargesit het.

Dan Maritz, woordvoerder, sê 'n span jong navorsers onder leiding van prof. Samson Sibeni Mashele en dr. Khajamohiddin Syed loop nou voor om oplossings te vind wat die maatskaplik-ekonomiese uitdagings wat die siektes vir veral die visserybedryf bied, te beëindig.

Syed sê miljoene seediere vrek jaarliks weens siektes wat deur parasiete veroorsaak word en dit het 'n regstreekse invloed op die visserybedryf.

Volgens hom is dit siemboeiend in die wetenskaplike wêreld dat die parasiete (componente) inhoue in die visserybedryf wêreldwyd saai en die lewensdruur van die diere aansienlik verkort.

Die navorsers het die P450 monooxygenase-proteïen in 13 patogeniese genome bestudeer en bevind dit kan as wegspringpunt gebruik word vir 'n middel om die swamagtige mikro-organismes te bestry.

Die navorsers ondersoek dit nou om oplossings te vind om seehulpbronne te beskerm.

Maritz sê hul (die navorsers se) werk beklemtoon die belangrikheid van die visserybedryf in die bevordering van gesonde lewe en die stryd teen armoede en honger.

Die uitkoms van die navorsing is reeds vir plasing in die Nature Publication Group Journal aanvaar.

■ Die wêreldbekende wetenskaplikes wat ook deel van die navorsingspan is, sluit in prof. David R. Nelson van die Universiteit van Tennessee, prof. Jae Hyuk Yu van die Universiteit van Wisconsin-Madison, en dr. Wanping Chen Huazhong van die Landbou-universiteit in China.

Die span navorsers aan die Sentrale Universiteit vir Tegnologie (SUT) wat die wegspringpunt vir 'n middel ontdek het om siektes wat deur parasiete veroorsaak word in seediere te bestry. Hier is van links, voor: dr. Khajamohiddin Syed; middel: Lorato Mongale, dr. Ntsaoli Malebo, Ponish Jawahierpersand en Mohammad Parvez; agter: prof. Sithwi Mashele, Mopeli Sello, Selao Baselemana, Lehlohonolo Qwanya, Mthakathi Ntsane, Thapelo Makhele, Euginiah Rampana en Richie Monyaki. Foto: MLUNGISI LOUW