CLONING AND CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS katG* GENE.

BY
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Dissertation Submitted In Partial Fulfillment For The Degree Of Master Of Science (Health Sciences) In Biomedicine

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CERTIFICATE

This is to certify that the dissertation entitled

"The cloning and chcracterization of *Mycobacterium tuberculosis* katG gene"

is the bonafide record of research work done by

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during the period June 2004 to March 2005

under my supervision.

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ABSTRACT

katG is the sole gene encoding for catalase peroxidase enzyme in *Mycobacterium tuberculosis* enabling its survival in the host macrophage. This same gene has been indicted to play an important role in forming the killing effect of isoniazid (INH), a major drug used in the treatment of tuberculosis (TB). It has been indicated that the formation of new generation drugs for TB will be connected to further understanding this interaction and the study of the structures of the katG gene.

The high molecular weight genomic DNA of *Mycobacterium tuberculosis* of the clinical strain MTB69/03 from HUSM has successfully been isolated through a modification of the method in D. van Soolingen *et. al.*. Two primers with extra sites for the excision sites of *Eco*R1 and *HindIII* were successfully used to produce a DNA product of 2252 base pairs. The product was then inserted into a pCR®2.1-TOPO® plasmid vector. The insert was confirmed by the use of both PCR and restriction enzymes.

The successfully confirmed inserted plasmids were then sent for sequencing.

ABSTRAK

katG merupakan gen tunggal bagi enzim katalase dan peroksidase dalam *Mycobacterium tuberculosis* dan berfungsi dalam membolehkan organisma tersebut hidup dalam makrofaj perumah. Gen tersebut turut memainkan peranan penting dalam kesam membunuh isoniazid (INH) yang merupakan drug penting dalam mengubati kes-kes tuberculosis (TB). Kajian telah menunjukkan bahawa penghasilan drug TB yang baru akan bergantung pada pemahaman tentang struktur katG dan interaksi katG dan INH.

DNA genomik *Mycobacterium tuberculosis* yang mempunyai berat molekul tinggi telah diasingkan dari strain klinikal MTB69/03 yang diperolehi dari HUSM. Teknik yang digunakan adalah modifikasi teknik yang ditunjukkan dalam D. van Soolingen *et. al.*. Dua primer yang mempunyai kawasan pemotongan untuk *Eco*R1 and *HindIII* telah digunakan dan telah berjaya menghasilkan produk DNA yang sebesar 2252 pasangan bes. Produk tersebut dimasukkan ke dalam vektor plasmid pCR®2.1-TOPO®. Konformasi kemasukkan primer yang betul dilakukan melaui PCR dan menggunakan enzim

Plasmid yang telah dipastikan betul dimasukkan produk kemudiannya dihantar untuk pendudukan.

1.0 INTRODUCTION

1.1 Tuberculosis(TB)

Tuberculosis is a disease that has long plagued man for many centuries. The earliest known name of this disease is Phthisis ("to waste") by the Greeks. It has also been known as consumption in the 1800s, wasting disease, and the white plague. This disease is known as the leading cause of death from a single infectious agent, with a prevalence of greater than 1.6 billion people (Whitney and Wainberg, 2002). Until the mid-1800s, people thought that tuberculosis, or TB, was hereditary. In 1865 a French surgeon, Jean-Antoine Villemin, proved that TB was contagious, and in 1882 a German scientist named Robert Koch discovered the bacterium that causes TB, *Mycobacterium tuberculosis.*

TB is caused by an organism called *Mycobacterium tuberculosis* (MTB) also sometimes called as the tubercle bacilli. It is a member of the genus mycobacterium which consists of members of the MTB complex and more than 80 species of non-tubercular mycobacteria including pathogenic, opportunistic and non pathogenic species (Soini et al., 2001). Mycobacteria can cause a variety of diseases. Some mycobacteria are called tuberculous mycobacteria because they cause TB or diseases similar to TB. These mycobacteria are MTB, *M. bovis*, and *M. africanum*. Other mycobacteria are called non-tuberculous mycobacteria because they do not cause TB. One common type of nontuberculous mycobacteria is *M. avium* complex. Non-tuberculous mycobacteria are not usually spread from person to person.

Tuberculosis (TB) is a communicable chronic granulomatous disease caused by MTB. It usually involves the lungs but may effect any other organ or tissue in the

body (Kumar, et *al.*,2003). Tuberculosis is normally spread through airborne transmission of droplet nuclei containing the MTB bacili. A majority of cases involves the lungs and is known as pulmonary tuberculosis where as extra pulmonary tuberculosis is due to the infection of other parts of the human body. About 80-90% of tuberculosis involves pulmonary tuberculosis and 10-20% involves extra pulmonary tuberculosis.

1.2 Epidemiology

There is archaeological evidence that tuberculosis has been occurring in human population for over 3000 years. This disease has been documented in analyses of mummies and skeletons which show deformities and lesions similar to that of a TB patient. Among the places that this evidence has been found include North Africa, Europe and even the Americas. There is no evidence of tuberculosis in sub-Saharan Africa, East Asia, or the Pacific until after contact was initiated with the Europeans, during the period of colonization (Aeras, 2005).

Tuberculosis grew to epidemic proportions in Europe beginning in the early 1600's as populations shifted to expanding cities and population densities increased. The worsening of the environmental factors due to pollution, the degradation of sanitation due to the mass translocation as well as the huge collection of individuals there lead to the spread of TB. Tuberculosis came to be the leading cause of death in Western Europe in the 18^{th} and early 19^{th} centuries (Whitney and Wainberg, 2002).

In the 19^{th} century there was a decline in tuberculosis that was accelerated by the development of effective chemotherapy for tuberculosis in the second half of the 20^{th} Century (Aeras,2005). One explanation of this decline is the rise in economic

conditions and the improvements in sanitation. Another reason might have been the segregation of individuals infected with TB from the general public via the use of sanatoriums. In the less industrialized parts of the world, the increase in tuberculosis occurred much later but was later halted by improving conditions in health and standards of living as well as the development and use dispersion of TB vaccines.

Unfortunately, this trend has been shown to be reversed. The World Health Organization (WHO) has reported that there are more than 8 million new cases of active disease each year and that 3 million people die annually from tuberculosis. The prevalence of TB is estimated to be over 1.6 billion people and is the cause of 7% of all death in developing countries (Rouse *et al.*, 1995). People in high risk groups in acquiring this disease are patients coinfected with HIV, prison inmates, and professionals who work with people in these high risk groups (O'Brien *et al.*, 1995).

A model by Dye C. in his publication Epidemiology, in Clinical Tuberculosis, 3rd Edition show that tuberculosis is on the increase and that the number of cases grew 1.7% per year from 1997 to 2000 and that with the present trends there will be 9-10 million new cases in 2010 (Aeras, 2005). The rise in TB infection could be attributed to four main factors. Firstly is the impact of HIV/AIDS in those countries were the epidemics co-exist. HIV infection rates among TB cases exceed 60% in South Africa, Zimbabwe and Zambia. Second, the impact of declining public health infrastructure in the world may via the worsening urban social and economic conditions result in the TB has resurged in the past decade (Rouse *et al.*, 1994). Third, the mixing of prison with civilian populations and lastly, recent improved surveillance and case reporting as other possible causes may be detecting the true prevalence of the disease.

Another further disturbing trend in the outbreak is the emergence of multi-drug resistant strains of the Mycobacterium, MDR-MTB. An important reason for this trend is patient non-compliance and delays in obtaining susceptibility data which still continue to be a hindrance to the elimination and instead further exacerbate the development of MDR-MTB (Peloquin, 1993). MDR TB has also been recognized as the cause of mortality for 70-90% of AIDS patients who develop TB (CDC, 2005) and the trend seems to be growing. WHO surveys report drug resistance in MTB isolates at rates of 1.7-36.9% in all 58 countries surveyed between 1996 and 1999. Mutants resistant to isoniazid (INH) is recognized as the most prevalent accounting for as much as 26% clinical MDR-MTB isolates in the USA (Banerjee, 1994).

1.3 Pathogenesis

Tuberculosis (TB) is a communicable chronic granulomatous disease caused by *Mycobacterium tuberculosis* (MTB) (Kumar *et al.*, 2003). It is spread via droplet nuclei in the air are about 1 to 5 microns in diameter (CDC, 2005) and can remain suspended in the air for several hours, depending on the environment. When a person inhales air containing particles expelled by an infectious person, most of the larger particles become lodged in the upper respiratory tract, where infection is unlikely to develop. However, the droplet nuclei may reach the alveoli, where infection begins. (Figure 2).

In, primary TB, an unsensitized individual or an individual never before exposed to the mycobacteria is infected by MTB. When MTB is inhaled and enters the lungs via alveoli, the bacterium becomes aggressively attacked and engulfed by

macrophages. But where other bacteria succumb to the attack, TB survives the macrophage with the aid of the "oxidative burst" mechanism which is due to the effects of superoxide dismutase and catalase-peroxidase which are enzymes that scavenge the oxygen radicals used as killing mechanisms by the macrophages. The TB will then be taken up by the alveolar macrophage and multiply in them. Once the mycobacteria gains entry into the macrophage, it inhibits normal bacteriacidal effects by manipulation of endosomal pH and arresting endosomal maturation (Kumar *et al.*, 2003).

A small amount of the TB bacilli will spread through the regional lymph nodes and then through the bloodstream to more distant tissues and organs including areas such as kidneys, brains and bone. Within 2 to 10 weeks after infection, the immune system usually intervenes. The immune response of hypersensitivity and resistance to the organism halts the multiplication of the tubercle bacilli and prevents further spread. In these instances, inflammatory conditions consolidate around the area of the MTB infection known as the Ghon focus which is also followed by tissue necrosis and scarring. This series of scarred tissue and consolidated cells are known as Gohn's complex. During this time, the patient usually doesn't suffer any symptoms other than a slight fever due to the infection. In this instance, the TB infection may resolve itself but usually, the complex may harbour viable MTB which may survive for years before being reactivated in secondary lesions.

In certain cases the disease may progress as progressive primary tuberculosis. This occurs when the MTB overcomes the defenses of the immune system and begin to multiply. This occurs in immunocompromised individuals or as the result of impairment in the defence mechanisms. The risk may be approximately 3 times greater with diabetes to more than 100 times greater for HIV infected

persons compared to those who do not have the disease (CDC, 2005). Other forms of this state are due to substance abuse, renal disease, neoplasia and hematological disorders.

Another scenario is when secondary TB infection or reactivation TB occurs. This is due to the reactivation of dormant primary lesion due to weakness of host resistance as well as exogenous reinfection of the host. In approximately 5% of persons who have been infected with MTB, TB disease will develop in the first or second year after infection. In another 5%, disease will develop later in their lives. The remaining 90% will stay infected, but free of disease, for the rest of their lives (CDC, 2005). This can occur within the lung or in other distant organs because of dissemination and seeding of the MTB into surrounding tissue via blood and the lymphatics. About 80-90% of TB involves pulmonary TB and 10-20% involves extra pulmonary TB (Kumar *et al.*, 2003). In extra pulmonary TB, the infection occurs in lungs, larynx, lymph nodes, brain, kidneys, bones or joints.

As the infection progresses, the infection causes localized damage to the tissue and then systemic damage. The majority of the infection which begins with pulmonary TB will cause progressive erosion into the bronchus of the lung forming cavities in the lung and the formation of fibrous tissue. Via the cavity the MTB can be disseminated into the air via the airways as well as via sputum. High amounts of sputum, mucoid in the beginning and later purulent will appear followed by hemoptysis.

Through the airways and through the lymphatic and vascular channels, the progressively multiplying MTB, may then cause military TB in which the MTB can then drain out into the blood stream and go to the heart where it is distributed to the arteries all over the body. Almost every organ of the body can then be seeded by

MTB foci. It is most prominent however in the liver, bone, spleen, adrenal, meninges, kidneys, fallopian tubes and epidedymis (Kumar *et al.*, 2003).

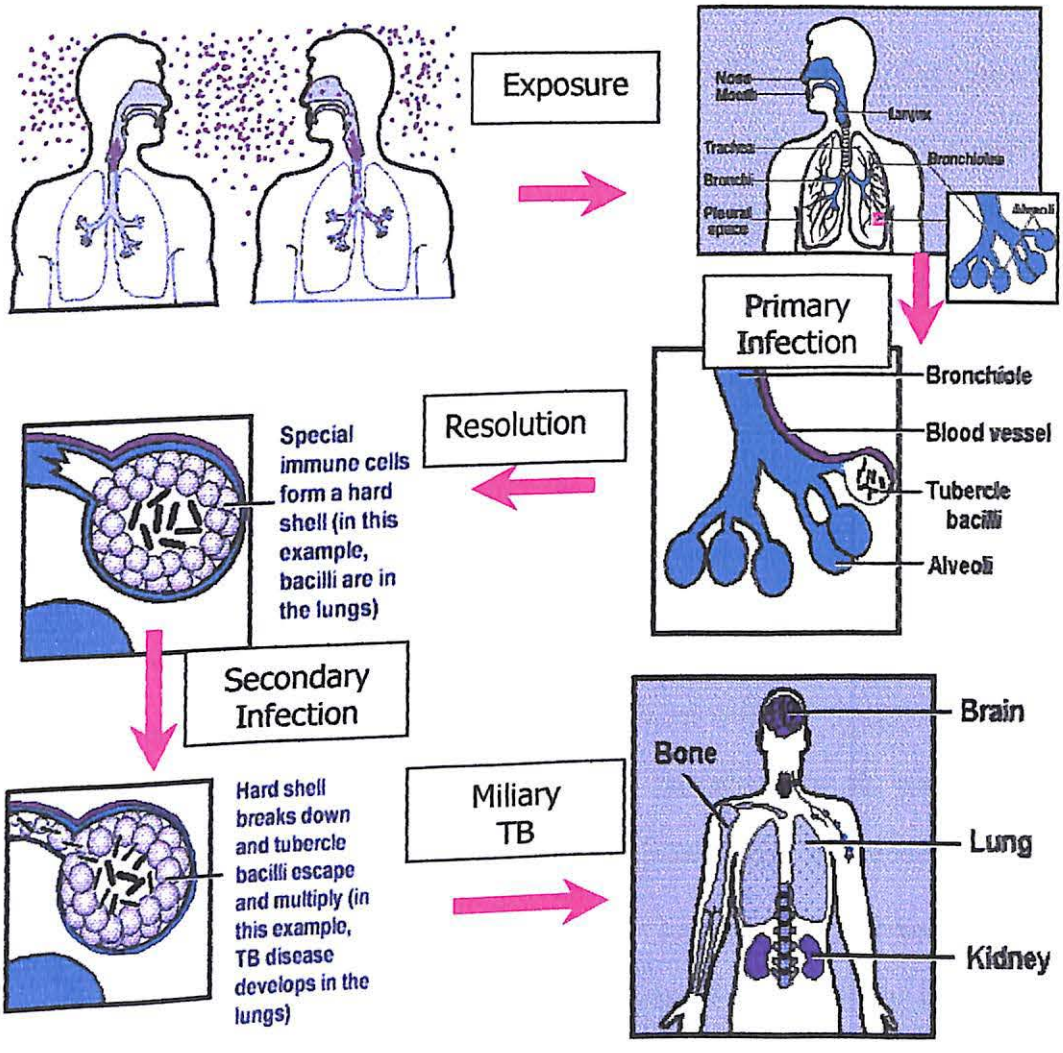

Figure 1: A chart depicting the pathogenesis of Mycobacterium Tuberculosis (CDC, 2005)

1.4 Control and Prevention

Because of this of the slow growth rate of the causative agent, isolation and identification, and drug susceptibility testing of this organism can take several weeks or longer (Soini *et al.*, 2001). A complete medical evaluation for TB includes a medical history, a physical examination, a Mantoux tuberculin skin test, a chest radiograph, and any appropriate bacteriologic or histologic examinations.

To date only 9 drugs have been approved by the FDA for the treatment of tuberculosis infections. Very few experimental drugs are available (Peloquin, 1993) and most of these drugs have been extensively used for the treatment of the disease. Due to the highly contagious and very stubborn nature of the disease, the treatment usually includes combinations of these drugs. The most prescribed form of treatment is that of the Arkansas regimen from the Unites States. It is a short course treatment is 6-9 months long and is only possible when isolates remain susceptible to both isoniazid and rifampin (Peloquin, 1993). The first line of drugs used in the fight against TB are isoniazid, rifampin, pyramidazole and ethambutol. The second line drugs or drugs used to complement these drugs include capreomycin, cycloserine, ethionamide, kanamycin, and the fluoroquinoles.

Initially, TB was in a decline especially in the developing countries. This was due mainly to the use of mass vaccination as well as improving public healthcare, treatment and the decline of poverty. Unfortunately, the trend shifted especially seen in the United States in the early 80s. This was mainly due to coinfection with the HIV virus (Haeym *et al.*, 1994). Another major reason for this rise was the appearance of multidrug resistant strains of MTB, MDR-MTB. These strains showed great resilience against the drugs used for the treatment against TB, having mortality rates significantly higher than that of drug sensitive strains (O'brien *et al.*, 1996).

In line with these trends, the WHO has been actively pursuing steps in eradicating this menace. It introduced DOTS (directly observed therapy, short course) and the Stop-TB Partnership, which encompassed proper handling of the disease, management and overall reporting of the disease through passive case-finding and treatment of the cases. It was based on the believe that proper

prescription and implementation of proper chemotherapy combining isoniazid, rifampin, pyrazinamide and ethambutol could eventually prove to be the answer (Haeym *et al.*, 1994). Unfortunately, patient non-compliance, rising poverty and rise in drug resistance continue to be the main problems in the treatment of the disease. A cure-rate of over 90% is possible with this approach and Dye notes that the average treatment success rate was 82% in 155 national programs (Aeras, 2005). However, coverage is far from complete and at the current rates of DOTS expansion the target of 70% case detection will not be reached until after 2010 (Aeres, 2005).

1.5 katG gene

katG is a gene with is 2223 bp in size which encodes the catalase peroxidase enzyme in MTB (Ng et al., 2004) and is imperative in conferring a selective advantage on the MTB by possibly enabling it to withstand hostile environments (Haeym *et al.*, 1995). This heme-containing enzyme is part of a group of proteins known as hydroperoxidase I (HPI) or catalase peroxisdase group (DeVito & Morris, 2002). The HPI protein is a bifunctional protein made out of catalase which turns hydrogen peroxide into water and oxygen ($2H_2O_2 \rightarrow H_20 + O_2$) Peroxidase on the other hand accepts protons to turn hydrogen peroxide into water ($H_2O_2 \rightarrow H_20$) (Haeym & Cole, 1997).

The significance of katG was highlighted by Zhang et al., when it was found that the deletion of this gene conferred isoniazid resistance,INH^R to *M tuberculosis* (O'Brien *et al.*, 1995). Isoniazid (INH) was first reported to be effective against MTB in 1952 and has been playing a pivotal role in the fight against TB since then (Quemard *et al.*, 1995). This drug is actually a prodrug, and has been shown to be

very effective against MTB with an MIC as low as 0.02μg/μl (Haeym *et al.*, 1992). The function is essential in the use of present treatments against TB. Although used extensively, the exact mechanism of action of this drug has still eluded us. Studies by J.A. Ainsa *et al.* (2001), and Wimpenny, (1967) that the enzyme produced by the katG gene activates the INH and is reacted once again before it becomes a form toxic to MTB. This new toxic form will then bind to the NADH and the complex then inactivates *inhA*, a gene that shares similar expression factors as katG. This is turn effects the action of the type II fatty acid synthase (FASII) impairing biosynthesis of mycolic acid and distrupts the cell wall production of the MTB (Ainsa *et al.*,2001). The katG protein has also been suggested to aid in the transport of INH into the cell (Rouse & Morris, 1995). Figure 3 contains an outline diagram of all the many theories put forth on the mechanisms of katG and its association with INH.

INH resistant strains of MTB make up 25% of most identified MTB strains. 60-70% of INH resistant strains have been linked to defects in katG gene. (Whitney & Wainberg, 2002) Patients infected with this strain showing this kind of resistance show poor prognosis with 46% of patients who undergo treatment having failures of relapses (Quennard *et al.*, 1995). Only about 50% will eventually be able to have negative sputum shedding of the virus (Goble, 1993).

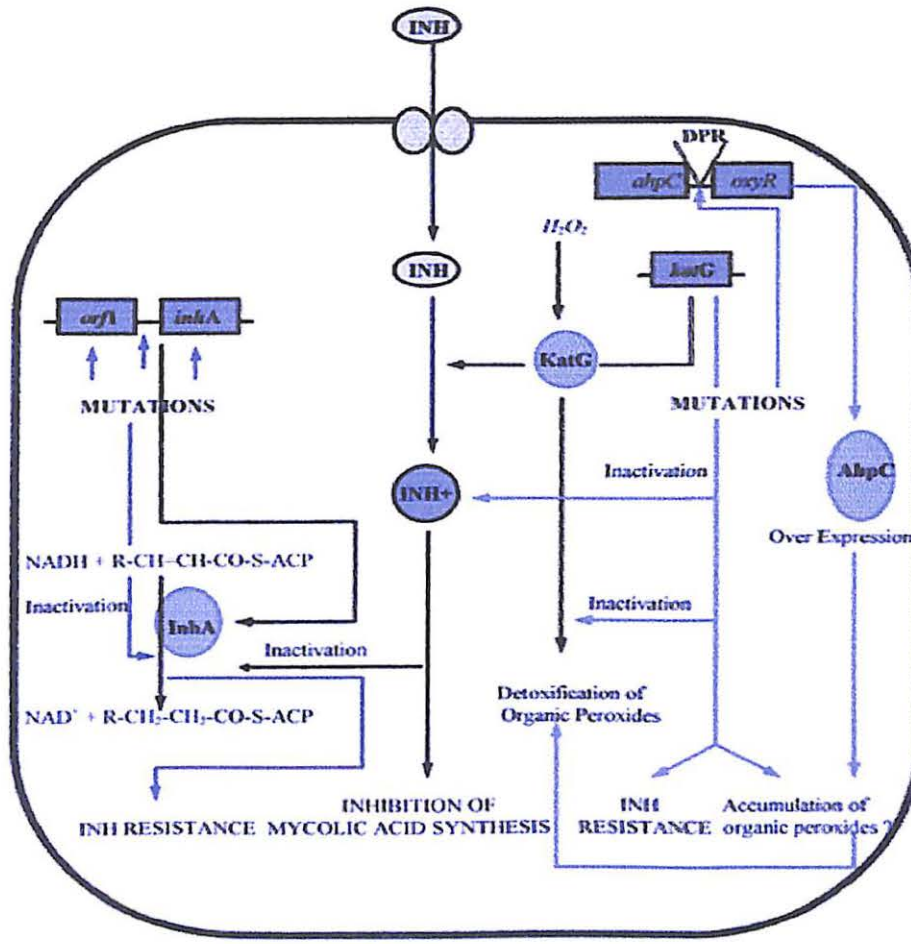

Figure 2: The effects of katG and katG mutagens on *Mycobacterium tuberculosis* survival with treatment with Isoniazid (INH).

1.6 Review of Literature

Zhang *et al.* (1992) demonstrated that INH resistance in some isolates was conferred by a total gene deletion of the katG gene which encodes catalase-peroxidase activity (O'Brien, 1995). This has led to the further understanding of the functions of isoniazid (INH) that had up till then been a mystery.

Haeym *et al.* (1993) in the characterization study of katG, concluded that the enzyme produced by katG interacts with INH and converts it into a toxic derivative

which is acted upon by a second unknown mechanism. This study was expanded by Ainsa *et al.* (2001), and Wimpenny, (1967) that each concluded that the enzyme produced by the katG gene activates the INH and undergoes another reaction before it becomes a toxic form to MTB. With this a basic theory on the workings on the INH action was founded.

Pym *et al.*, (2001) in their study of the regulation and virulence of *katG* and its promoters concluded that antimycobacterial activity of INH is limited by the rate of activation and not interaction with other cellular processes. Thus, INH analogues was the rational strategy for a new generation INH-based drugs.

DeVito & Morris (2002) in studying the structures of the katG gene found that a rational design of INH-based analogues is difficult because katG protein crystal structure and tertiary and quartenary structural determinants still remained unknown.

Recently the genome of the MTB laboratory strain H37RV was completely sequenced (GenBank accession no. NC 000962) and allows for better research and understanding of the relationship of the genes.

Due to these findings, it can be concluded that the structure of katG is an important determinant in finding an alternative cure for TB in the near future. The isolation and expression of the katG gene is possible and has been performed. The molecular structures and structural determinants can now be studied to further expand on the present knowledge on the interactions of katG with isoniazid and the killing effects. This would allow better understanding on ways to deal with the TB epidemic and enable the rational design of INH-based derivatives/analogues which will be more effective than INH.

1.7 Objectives

Although the katG gene has been cloned and the sequence is known, the structure of this enzyme, however, has yet to be elucidated. The objective of this study is to clone the katG gene which will later be used to express the enzyme for study of the protein structure by X-ray crystallisation.

1.7 Flowchart of Project

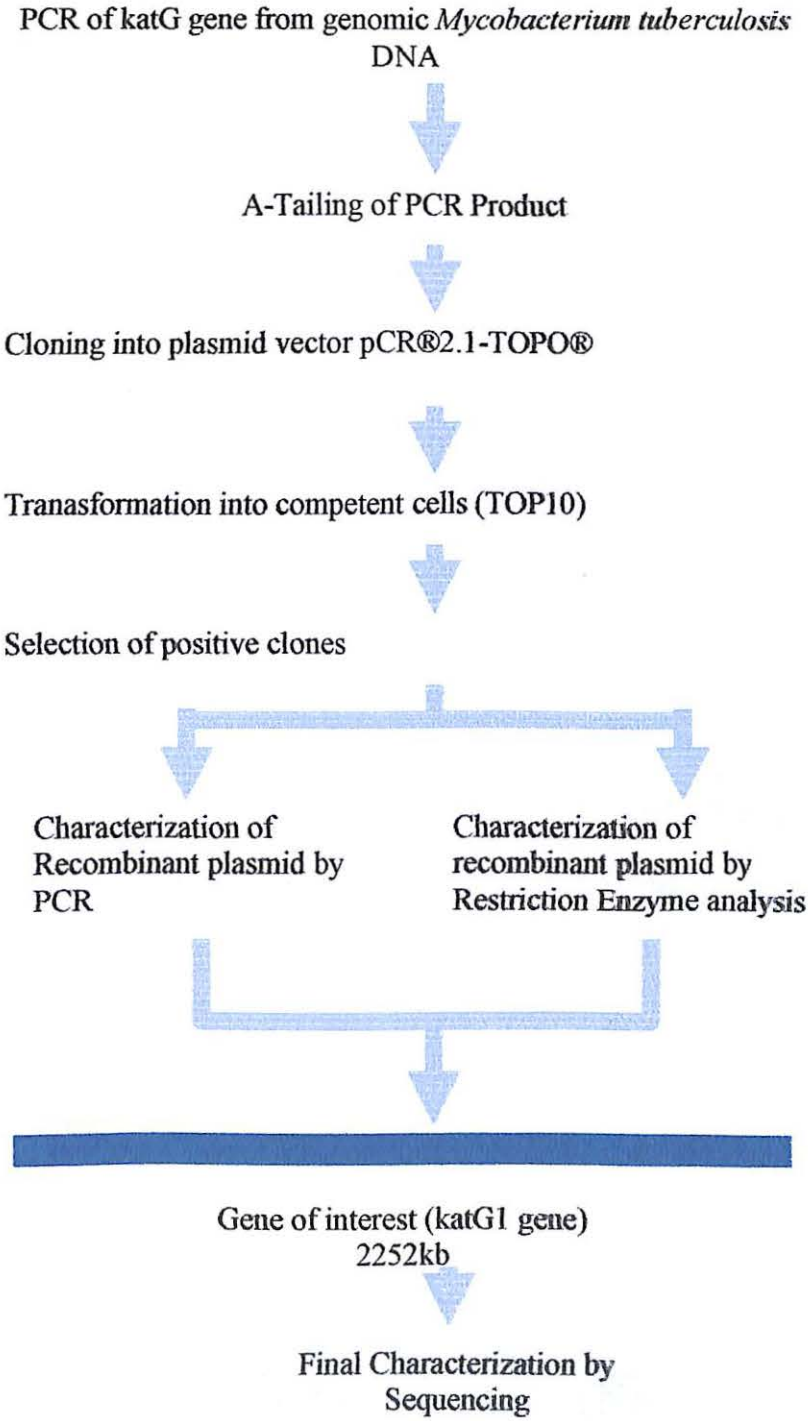

Figure 3: A Flowchart to outline the steps involved to be accomplished in the experiment

2.0 MATERIALS

2.1 Reagents and Chemicals

No	Reagents and Chemicals	Supplier
1	7H9 Middle Brooke	Pifco Lab, USA
2	OADC enrichment media	Becton Dickinson & Co., USA
3	Sodium pyruvate 100mM/L	Bio-Rad, USA
4	Tris base	Amres Co. ®, USA
5	EDTA	Promega, USA
6	Lysozymes	Merck, Germany
7	Sodium dodecyl sulphate	Bio-Rad, USA
8	Protinase k	Invitrogen, USA
9	Sodium chloride (NaCl)	Merck, Gemany
10	N-acetyl-N,N,N-trimethylammonium bromide (CTAB)	Sigma, USA
11	Isoamyl-alcohol	BDH Analar
12	Chloroform	M&B Laboratory
13	Isopropanol	Merck, Germany
14	70% ethanol	Merck, Germany
15	Sodium Hydroxide (NaOH)	Merck, Germany
16	Glacial acetic acid	Bio-Rad, USA
17	Agarose	Promega, USA
18	Orange G	Sigma, USA

19	Ethidium bromide	Sigma, USA
20	Sucrose	AnalaR®, Germany
21	Tryptone	Pronidasa, Madrid
22	Yeast extract	Pronidasa, Madrid
23	*E. coli* (TOP10), -70°C	Invitrogen, USA
24	Magnesium chloride ($MgCl_2$)	AnalaR®, Germany
25	Calcium chloride ($CaCl_2$)	AnalaR®, Germany
26	Glycerol 10%	Sigma, USA
27	Kanamycin	Promega, USA
28	X-Gal	Promega, USA
29	Lambda DNA/ Hind III marker	Promega, USA
30	100bp DNA ladder	Promega, USA
31	Blue/Orange 6x Loading Dye	Promega, USA

2.2 Kits

No.	Kits	Supplier
1	*Taq* DNA Polymerase: *Taq* DNA Polymerase 10x Reaction Buffer $MgCl_2$ dNTP mix	Fermentas, USA
2	*Pfu* DNA Polymerase: *Pfu* DNA Polymerase 10x Reaction Buffer	Fermentas, USA

	$MgSO_4$ dNTP mix	
3	*Taq* DNA Polymerase: *Taq* DNA Polymerase 10x Reaction Buffer $MgCl_2$ dNTP mix	Promega, USA
4 5	QIA Quick® Gel Extraction kit TOPO® Cloning Reaction pCR®2.1-TOPO® vector salt solution	QIAGEN, USA Invitrogen, USA
6	QIAprep® Miniprep	QIAGEN, USA
7	*EcoRI* Restirction Enzyme *EcoRI* restriction enzyme 10x buffer	Fermentas, USA

2.3 Equipment

No.	Equipment	Supplier
1	Dragon 3002 Analytical Balance	Mettler Toledo, Canada
2	Cyberscan 1000 pH meter	Eutech, UK
3	TBVX Hexomix Water Bath	Heto-High Technology, Scandinavia
4	Innova 4080 Rotary Shaker	New Brunswick Scientific Co. Inc. USA

5	Pipettes	Eppendorf, Germany
6	Eppendorf Fixed Angle Rotar Microcentrifuge	Eppendorf AG, Germany
7	Refrigerated Centrifuge 5810R	Eppendorf, Germany
8	Gene AMP PCR System 9700 Thermal cycler	Applied Biosystems,USA
9	PTC-200 DNA Engine (Peltier Thermal Cycler)	MJ Research, USA
10	Super Showerwave Microwave Oven	Sanyo,
11	Agarose Gel Electrophoresis Apparatus	CBS Scientific Co.,USA
12	Elite300Plus	Wealtec, USA
13	UVP Bioimaging UVP Image Analyzer	Lab Companion,USA
14	UV-25 UV Transilluminator	Hoefer, UK
15	Nanodrop ™ ND-100 Spectrophotometer	Nnanodrop™,USA
16	Shaking Incubator SI600	Lab Companion, USA
17	Microbiological Incubator	Binder, Germany
18	Lambda EZ 150 UV-Vis Spectrophotomer	Perkin Elmer,USA

3.0 PREPARATION OF REAGENTS

3.1 Preparation of 7H9 Middle Brooke broth

7H9 Middle Brooke broth was prepared by dissolving 2.35g of the powdered broth in 450mL deionised distilled water. 19.09mL sodium pyruvate 100 mM/L which has been filtered prior to use is then pipetted. The solution is then mixed thoroughly and aliquoted into flasks. 50 mL OADC enrichment media is then added to the solution prior to use.

3.2 Preparation of 10xTE buffer

10x TE buffer was prepared from the adding 12.1g of Tris in pH8.0 with 3.7g 1mM EDTA. The pH was adjusted with sodium hydroxide. Deionised distilled water was then added to make up the solution to a level of 100 mL. The solution was autoclaved at 121°C for 15 minutes and stored at room temperature.

3.3 Preparation of 10mg/mL lysozymes

The lysozymes are prepared into concentrations of 10mg/mL by adding 0.1g of the powdered lysozymes provided with 10 mL deionised distilled water. The solution is then thoroughly mixed and filter sterilised prior to use.

3.4 Preparation of 10% SDS (sodium dodecyl sulphate)

The 10% SDS is by adding 10g of sodium dodecyl sulphate in powder form into 100mL of distilled water. The solution is then dissolved by heating at 65°C for 20 minutes. The solution is not autoclaved. The solution may then be kept at room temperature for not longer than 1 month.

3.5 Preparation of Protinase k (1mg/mL)

Protinase k (1 mg/mL) was prepared by dissolving 10mg of protinase k in 100mL distilled water. The solution was then mixed properly and filter sterilized. The solution was then stored in small aliquots at -20°C for not more than 1 year.

3.6 Preparation of CTAB/NaCl solution

CTAB/NaCl solution is prepared by dissolving 4.1 g of sodium chloride and 10 g N-acetyl-N,N,N-trimethylammonium bromide (CTAB) in 80mL of deionised distilled water. The solution is then stirred on a heater block. Deionised distilled water is then added up to 100 mL. The complete solution is then autoclaved at 121°C for 15 minutes.

3.7 Preparation of isoamyl-alcohol (24:1)

Isoamyl and alcohol in of 24:1 is prepared by adding 4 mL of the isoamyl-alcohol with 9 mL of chloroform. The solution was then mixed thoroughly and then stored in a dark bottle.

3.8 Preparation of Sodium Hydroxide (NaOH) 1M

1M sodium hydroxide was prepared by dissolving 12g of NaOH in 300 mL deionised distilled water. The solution was autoclaved at 121°C for 15 minutes and stored at room temperature.

3.9 Preparation of 50x TAE (Tris-Acetate) Buffer

The 50x TAE buffer is prepared by adding 121g of Tris base in 50 mL 0.5M EDTA at pH 8.0. 1M Sodium hydroxide is added to adjust the pH to 8.0. 28. 55 mL of glacial acetic acid is then added to the solution. The solution is then mixed on a heated plate with a stirrer. Deionised distilled water is then added to the solution to make up to 500mL. The solution is thoroughly mixed with a stirrer. The media was autoclaved at 121°C for 15 minutes. The solution is used as a stock solution for the preparation of 1x TAE buffer which is prepared by adding 10mL 50x TAE buffer with deionised distilled water to make up 500 mL.

3.10 Preparation of Orange G loading buffer

Orange G is prepared by diluting 0.0625g Orange G in powder form in 25 mL distilled water. 10g sucrose is then added to the solution. The solution is then thoroughly mixed.

3.11 Stock Solution of Ethidium bromide

Ethidium bromide was prepared by diluting 1g of ethidium bromide into 1000 mL deionised distilled water. The solution was then stored in a dark bottle with warning signs.

3.12 Preparation of Luria-Bertani (LB) agar

LB agar was prepared by dissolving 15g tryptone, 5g of yeast extract, 10g of NaCl and 15g of agar into 750 mL of deionised distilled water. The pH was adjusted to 7.2 with 1M NaOH. Deionised distilled water was added to make the final volume of 1000 mL. The media was autoclaved at 121°C for 15 minutes. The media was cooled down before the apropiate antibiotic was added. 10 µL of kanamycin 50 mg/ µL may be added to produce LB agar with kanamycin. The agar was stored in the cold room.

3.13 Preparation of Luria Bertani (LB) broth

LB broth was prepared by 15g tryptone, 5g of yeast extract and 10g of NaCl into 750 mL of deionised distilled water. The pH was adjusted to 7.2 with 1M NaOH. Deionised distilled water was added to make the final volume of 1000 mL. The media was left to cool before being aliquoted into universal bottles. The media was autoclaved at 121°C for 15 minutes and stored at room temperature.

3.14 Preparation of Competent cells

E. coli competent cells were prepared by first culturing the cells in 10 mL of LB (Luria-Bertani) broth. The culture was then incubated at 37°C with vigorous shaking at 180 rpm horizontally in the shaking incubator overnight. 100 µL of the overnight culture was then aliquoted and inserted into another 10 mL of LB broth. The culture was incubated at 37°C in the shaking incubator. The optical density of the culture was observed every half an hour with a spectrophotometer at wavelength of 600nm. When the culture showed an OD of 0.4-0.5, the culture was then chilled on ice for 10 minutes. The culture was then centrifuged at 3000rpm for 10 minutes at a temperature of 4°C. The supernatant produced was discarded and the pellets were resuspended with 6mL of cold 100 mM magnesium chloride ($MgCl_2$). The cells were then chilled on ice for 45 minutes. The cells were centrifuged again at 3000rpm for 10 minutes at a temperature of 4°C. The supernatant produced was once again discarded. The remaining pellets were resuspended in 400 mL of cold 100 mM calcium chloride ($CaCl_2$). 60 µL of 10% glycerol was then added to the

cells. 50 µL of the suspension was then aliqouted into 1.5 mL microcentrifuge tubes and stored at -70°C.

3.15 Preparation of Kanamycin stock (50mg/mL)

Kanamycin stock was prepared by adding 10 mg of kanamycin sulphate into 10 mL deionised distilled water. The mixture was filtered using 0.2 µm filter memebrane prior to use. The solution was aliquoted for into quantities of 1mL and stored in microcentrifuge tubes at -20°C.

3.16 Preparation of 100bp DNA ladder

100bp DNA ladder was prepared by adding 50 µL of 100bp DNA ladder with Blue/Orange 6x loading buffer in a microcentrifuge tube. The mixture was then mixed properly and stored at 4°C.

3.17 Preparation of Lambda DNA/ Hind III marker

Lambda DNA/ Hind III marker was prepared by adding 50µ L of Lambda DNA/ Hind III marker with Blue/Orange 6x loading buffer in a microcentrifuge tube. The mixture was then mixed properly and stored at 4°C.

4.0 GENERAL METHOD

4.1 Isolation of high molecular weight genomic DNA from mycobacteria.

This method was a modified version of the isolation technique described by van Soolingen *et al.* (1991). *Mycobacterium tuberculosis* samples were first cultured in 7H9 broth and incubated at 37°C for 2 weeks. 1.0mL of each culture was placed into a microcentrifuge tube. The sample is then heated at 80°C for 20 minutes in the water bath. This step was used to kill the cells in the sample. The culture was spun in a microcentrifuge at 12000 rpm for 5 minutes. The resulting supernatant was then discarded with the use of a micropippette. The pellet was then resuspended with 500µL 1x TE buffer by means of vortexing. 50 µL 10mg/mL lysozymes was then added to the solution. The sample was then mixed via vortexing and then incubated at 37°C for 1 hour in the water bath. This process allowed the lysozyme to break the cell walls. 70µL of 10% SDS (sodium dodecyl sulphate) and 6 µL of 10mg/mL proteinase K were then added to the sample to digest the proteins in the sample. The sample was then vortexed for about 10 seconds to mix and then incubated at 65°C for 10 minutes. 100 µL of 5M NaCl was then added to the sample and mixed by vortexing for 30 seconds. 80 µL of prewarmed CTAB/NaCl solution was added to the sample. The sample was then mixed via vortex till the sample became creamy or milky white. The sample was then incubated at 65°C for 10 minutes. An equal amount of chloroform/isoamyl chloroform (24:1) which equals to 700 µL was then added to the sample and then mixed with the use of a vortex for 10 seconds. The sample was then centrifuged at room temperature for 5 minutes at 12000 rpm. 600