Subcloning of *Mycobacterium tuberculosis* KatG gene in bacterial expression system

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Dissertation submitted in partial fulfillment
of the requirements for the degree
of Bachelor of Health Sciences (Biomedicine)

2006

CERTIFICATE

This is to certify that the dissertation entitled

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ACKNOWLEDGEMENT

Praised be to Allah, the Cherisher and Sustainers of the worlds, who have given me the strength to finally complete my dissertation. Peace and Blessing be upon the Seal of the Prophets, Muhammad. May peace and blessing be upon him and his companions.

I wish my appreciation and thanks to my Supervisor, Prof. Zainul Fadzirudin Zainuddin for his assistance and guidance for the duration of this study and for his great patience and effort in developing my knowledge, experience and understanding especially in the field of research and laboratory management.

I also wish thanks to Dr. Shaharum Shamsuddin as my Co-Supervisor for her continuous advice and encouragement to point me on the right path towards this molecular biology field.

Special thank also to Ms Khairul Ezani MSc. Student who have spend her time to teach and advice me about laboratory works and also help me in troubleshooting if my works goes wrong.

The deepest thanks also to Mr Mohd Nasir Bin Mohd Aris, Mrs Zainab Bt Mat Yasin and all of my siblings who has gave the full of moral and financial support to me for finished my research.

I would also like to thank the Science Officers, Technologists, Research Officers, Masters and Ph.D. students who were at the Advanced Molecular Biology Laboratory who helped a great deal to solve the problems and doubts I

had while embarking on this research project, namely Mr Venugopal, Mrs Azizah, Ms Arnida, Mr Muhammad, Ms Noor Hafiza, Mrs Asma and and persons unnamed. These individual thought a lot on daily practices in molecular laboratory and widen my knowledge about possibilities of career in this very field.

Lastly I would like to thank all the individuals mentioned above again for helping me to acquire the very basic of skills in molecular works and the talent to troubleshoot.

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ABSTRACT

KatG is the gene in *Mycobacterium tuberculosis* which responsible for encoding of catalase peroxidase enzyme to enable its survival in host macrophage. *KatG* also reported to play an important role in enabling the killing effect of the anti-tuberculous pro-drug, isoniazid (INH). The mutation of this gene has been reported to create resistancy of *Mycobacterium tuberculosis* toward INH treatment. Since the killing effect of the drug has a significant relation with this gene, the structure and properties of *KatG* should be study for further information.

*Eco*RI restriction enzyme was found to produce 2252 bp of DNA product. The gene was then sliced out from pCR®2.1-TOPO by using *Eco*RI restriction enzyme and ligated into a high-level protein expression vector, pRSET for expression assay. The recombinant sequenced has been confirmed by *Eco*RI-*Pvu*II restriction analysis. The gene has also been sequenced by cycle sequencing and the primers are M13F, M13R, KG3 and KG5.

1 Introduction

1.1 Tuberculosis

Tuberculosis (commonly shortened to TB) is an infectious disease which caused by infection with an acid fast bacilli named *Mycobacterium tuberculosis.* It is most commonly affects lungs (pulmonary TB) but can also affect the central nervous system (meningitis), lymphatic system, circulatory system (Miliary tuberculosis), genitourinary system, bones and joints.

TB is a disease rich in paradoxes. It was one of the first disease for which the causative agent was identified, and one of the first for which a vaccine was developed (Calmette *et.al.,* 1929 and Koch,R.,1982). Antibiotics effective against *M.tuberculosis* have been widely available for a half century (Iseman, *et.al.,* 2002). Yet it remains one of the great killer's infectious diseases in the world: HIV/AIDS kills 3 million people each year, TB kills 2 million, and malaria kills 1 million (WHO 1998).

Most recently, the human immunodeficiency virus (HIV) epidemic caused the number of new TB cases to explode (Aaron *et.al.,* 2004). Multiple drug resistant strains of TB (MDR-TB) are emerging. The World Health Organization declared TB a global health emergency in 1993.

1.1.1 History of tuberculosis

Tuberculosis has been present in human since antiquity, as the origins of the diseases are in the first domestication of cattle. Tubercular decay has been found in the fragments of the spinal column from Egyptian mummies from 2400 B.C (*Mathew Sarrel*).

Around 460 BC Hippocrates identified "phthisis" (a Greek term for consumption) as the most widespread disease of the time which was almost always fatal. He even went so far as to warn other doctors not to visit patient in late stage of the disease due to the danger of catching it themselves.

In the 17th century people began to describe and record the exact pathology of the disease. In 1679, Sylvius describe the actual tubercles as a characteristic change in the lungs and other parts of the body in consumptive patients in his publication named *Opera Medica.* He also described their progression to abscesses and cavities.

In 1720, the English physician Benjamin Marten was the first to conjecture, in his publication, *A New Theory of Consumption*, which TB could be caused by "wonderfully minute living creatures", which, once they had gained a foothold in the body, could generate the lesions and symptoms of the disease. He stated, moreover, "It may be therefore very likely that by an habitual lying in the same bed with a consumptive patient, constantly eating and drinking with him, or by very frequently conversing so nearly as to draw in part of the breath he emits from the Lungs, a consumption may be caught by a sound person...I imagine that slightly conversing with consumptive patients is seldom or never sufficient to catch the

disease." For the early eighteenth century, Dr. Marten's writings display a great degree of epidemiological insight.

The bacillus-causing tuberculosis, *Mycobacterium tuberculosis*, was described on March 24, 1882 by Robert Koch. He received the Nobel Prize in physiology or medicine in 1905 for this discovery of a staining technique that enabled him to see *Mycobacterium tuberculosis.*

The first genuine success in immunizing against tuberculosis developed from attenuated bovine strain tuberculosis by Albert Calmette and Camille Guerin in 1906 was BCG (Bacillus of Calmette and Guerin). It was first used on humans on July 18, 1921 in France, although national arrogance prevented its widespread use in either the USA, Great Britain, or Germany until after World War II.

In fact, the chemotherapy of infectious diseases, using sulfonamide and penicillin, had been underway for several years, but these molecules were ineffective against Mycobacterium tuberculosis. Since 1914, Selman A. Waksman had been systematically screening soil bacteria and fungi, and at the University of California in 1939 had discovered the marked inhibitory effect of certain fungi, especially actinomycete, on bacterial growth. In 1940, he and his team were able to isolate an effective anti-TB antibiotic, actinomycin; however, this proved to be too toxic for use in humans or animals.

Success came in 1943. In test animals, streptomycin, purified from *Streptomyces griseus*, combined maximal inhibition of M. tuberculosis with relatively low toxicity. On November 20, 1944, the antibiotic was administered for the first time to a critically ill TB patient. The effect was almost immediately impressive. His advanced disease was visibly arrested, the bacteria disappeared

from his sputum, and he made a rapid recovery. The new drug had side effects - especially on the inner ear - but the fact remained, M. tuberculosis was no longer a bacteriological exception, it could be assailed and beaten into retreat within the human body.

A rapid succession of anti-TB drugs appeared in the following years. These were important because with streptomycin monotherapy, resistant mutants began to appear with a few months, endangering the success of antibiotic therapy. However, it was soon demonstrated that this problem could be overcome with the combination of two or three drugs.

1.1.2 The bacterium

The cause of tuberculosis, Mycobacterium tuberculosis (MTB), is a slow-growing aerobic bacterium that divides every 16 to 20 hours. This is extremely slow compared to other bacteria, which tend to have division times measured in minutes (among the fastest growing bacteria is a strain of E. coli that can divide roughly every 20 minutes). It is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although it contains peptidoglycan in their cell wall. If a Gram stain is performed, it stains very weakly Gram-positive or not at all. It is a small rod-like bacillus which can withstand weak disinfectants and can survive in a dry state for weeks but, spontaneously, can only grow within a host organism (in vitro culture of M. tuberculosis took a long time to be achieved, but is nowadays a normal laboratory procedure).

MTB is identified microscopically by its staining characteristics: it retains certain stains after being treated with acidic solution, and is thus classified as an

"acid-fast bacillus" or "AFB". In the most common staining technique, the Ziehl-Neelsen stain, AFB are stained a bright red which stands out clearly against a blue background. Acid-fast bacilli can also be visualized by fluorescent microscopy, and by auramine-rhodamine stain.

The M. tuberculosis complex includes 3 other mycobacterium which can cause tuberculosis: M. bovis, M. africanum, and M. microti. The first two are very rare a cause of disease and the last one does not cause human disease. Nontuberculous mycobacteriums (NTM) is other mycobacterium (besides M. leprae which causes leprosy) which may cause pulmonary disease resembling TB, lymphadenitis, skin disease, or disseminated disease. These include Mycobacterium avium, M. kansasii, and others.

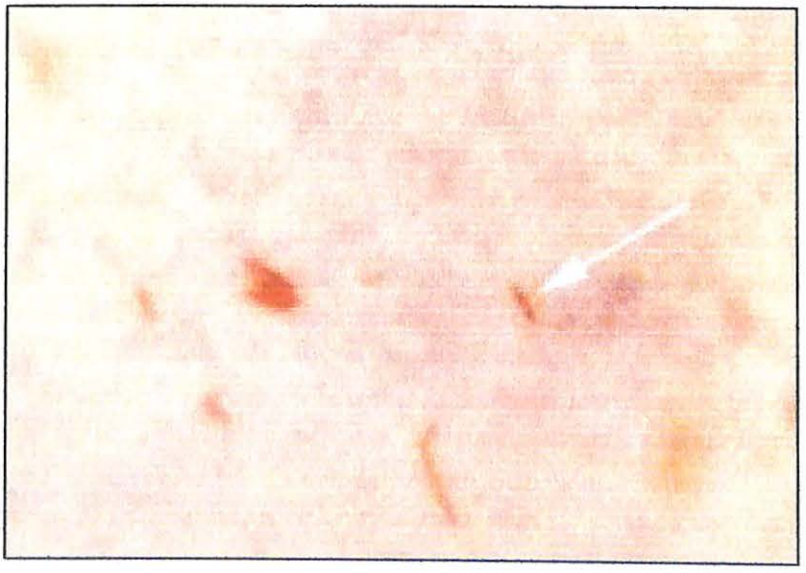

Figure 1.1: Acid-fast bacilli (AFB) (shown in red) are tubercle bacilli

1.1.3 Pathogenesis

1.1.3.1 Transmission

TB is spread through aerosol droplets which are expelled when persons with active TB disease cough, sneeze, speak, or spit. Close contacts (people with prolonged, frequent, or intense contact) are at highest risk of becoming infected (typically 22 percent infection rate but everything is possible, even up to 100%). A person with untreated, active tuberculosis can infect an estimated 20 other people per year. Others at risk include foreign-born from areas where TB is common, residents and employees of high-risk congregate settings, health care workers who serve high-risk clients, medically underserved, low-income populations, high-risk racial or ethnic minority populations, children exposed to adults in high-risk categories, and people who inject illicit drugs.

Transmission can only occur from people with active TB disease (not latent TB infection).

The probability of transmission depends upon: infectiousness of the person with TB (quantity expelled), environment of exposure, duration of exposure, and virulence of the organism.

The chain of transmission can be stopped by isolating patients with active disease and starting effective anti-tuberculous therapy.

1.1.3.2 Infection

TB infection begins when TB bacilli reach the pulmonary alveoli, and infect alveolar macrophages, where the mycobacterium replicates exponentially. Bacteria are picked up by dendritic cells, which can transport bacilli to local (mediastinal)

lymph nodes, and then through the bloodstream to the more distant tissues and organs where TB disease could potentially develop: lung apices, peripheral lymph nodes, kidneys, brain, and bone.

Tuberculosis is classed as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T lymphocytes secrete cytokine such as interferon gamma, which activates macrophages and make them better able to fight infection. T lymphocytes can also directly kill infected cells.

Importantly, bacteria are not eliminated with the granuloma, but can become dormant, resulting in a latent infection. Latent infection can be diagnosed only by tuberculin skin test, which yields a delayed hypertype sensitivity response to purified protein derivatives of M. tuberculosis in an infected person.

Another feature of the granulomas of human tuberculosis is the development of cell death, also called necrosis, in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous necrosis.

If TB bacteria gain entry to the blood stream from an area of tissue damage they spread through the body and set up myriad foci of infection, all appearing as tiny white tubercles in the tissues. This is called miliary tuberculosis and has a high case fatality.

In many patients the infection waxes and wanes. Tissue destruction and necrosis are balanced by healing and fibrosis. Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are in continuity with the air passages bronchi. This material may therefore be coughed up. It contains living bacteria and can pass on infection.

Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Affected areas are eventually replaced by scar tissue.

1.2 KatG gene

KatG gene is a gene that responsible to encode the catalase peroxidase enzyme in MTB which has 2223 bp in size (NG *et al.*, 2004). This heme-containing enzyme is part of a group of proteins known as hydroperoxidase I (HPI) or catalase peroxidase group (DeVito & Morris, 2002). The HPI protein is a bifunctional protein made out of catalase which turns hydrogen perixide into water and oxygen ($2H_2O_2 \rightarrow H_2O + O_2$). Peroxidase on the other hand accepts protons to turn hydrogen peroxides into water ($H_2O_2 \rightarrow H_2O$) (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 *Mycobacterium 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 pro-drug, 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 this drug has still eluded us. Studies by J.A. Ainsa *et al.* (2001), and Wimpenny,(1967) that the katG gene activates the INH and reacted once again before it become a form toxic to MTB. This new toxic form will then bind to the NADH and the complex then inactivates *inhA,* a gene that share similar expression factor as katG. This is turn effects the action of the type II fatty acid synthase (FASII) impairing biosynthesis of mycolic acid and disrupts the cell wall production of the MTB (Ainsa *et al.*, 21001).

1.3 Review of literature

The usefulness of isoniazid (INH), a key component of short-course chemotherapy of tuberculosis, is threatened by the emergence of drug-resistant strains of *Mycobacterium tuberculosis* with mutations in the katG gene (Espinal *et al.,* 2001). The studies of Stover *et al.,* 2000 shows that resistance of *M. tuberculosis* to antimicrobial drugs is the consequence of spontaneous mutation in genes that encode either the target of the drug, or enzymes that are involved in drug activation. Heym *et al.,* 1994; and Zhang *et al.,* 2000 has described resistance-associated points mutation, deletions, or insertions for all first line drugs (isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin), and for several

second line and newer drugs (ethionamide, fluoroquinolones, macrolides, nitroimidazopyranes).

Middlebrook *et al.,* 1954 has shown that isoniazid-resistant clinical isolates frequently lost its catalase and peroxidase activity. However, the association of this enzyme with isoniazid activation was not proven until the early 1990's, when the primary mycobacterial catalase-peroxydase gene (katG) was cloned and sequenced (Zhang *et al.,* 1992). Another study also has shown that mutation in this gene are found in 42- 58 % of isoniazid-resistant clinical isolates (Zhang *et al.,* 2000 ; Ramaswamy *et al.,* 1998).

Abal *et al.,*2002 shows that 69% of the isoniazid-resistance from clinical isolates have mutation on katG gene where where Ser 315 is replaced by Thr (S315T) (Ahmad *et al.,* 2004).

Due to these findings, it can be concluded that the structure of katG is an important determinant in finding the way on TB preventions. The isolation and expression of the katG gene is possible and has been performed. The molecular structure can be study now to expand the knowledge on the relationship of katG with isoniazid and the killing effect.

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1.4 Objectives

The main objective of this study is to characterize the katG gene by sequencing and restriction enzyme analysis. Since the sequence of katG has been known, the gene then ligated into the expression vector. The ligated katG into expression vector will later can be used to express the enzyme to study the protein structure by X-ray crystallography.

1.5 Flow chart of the study

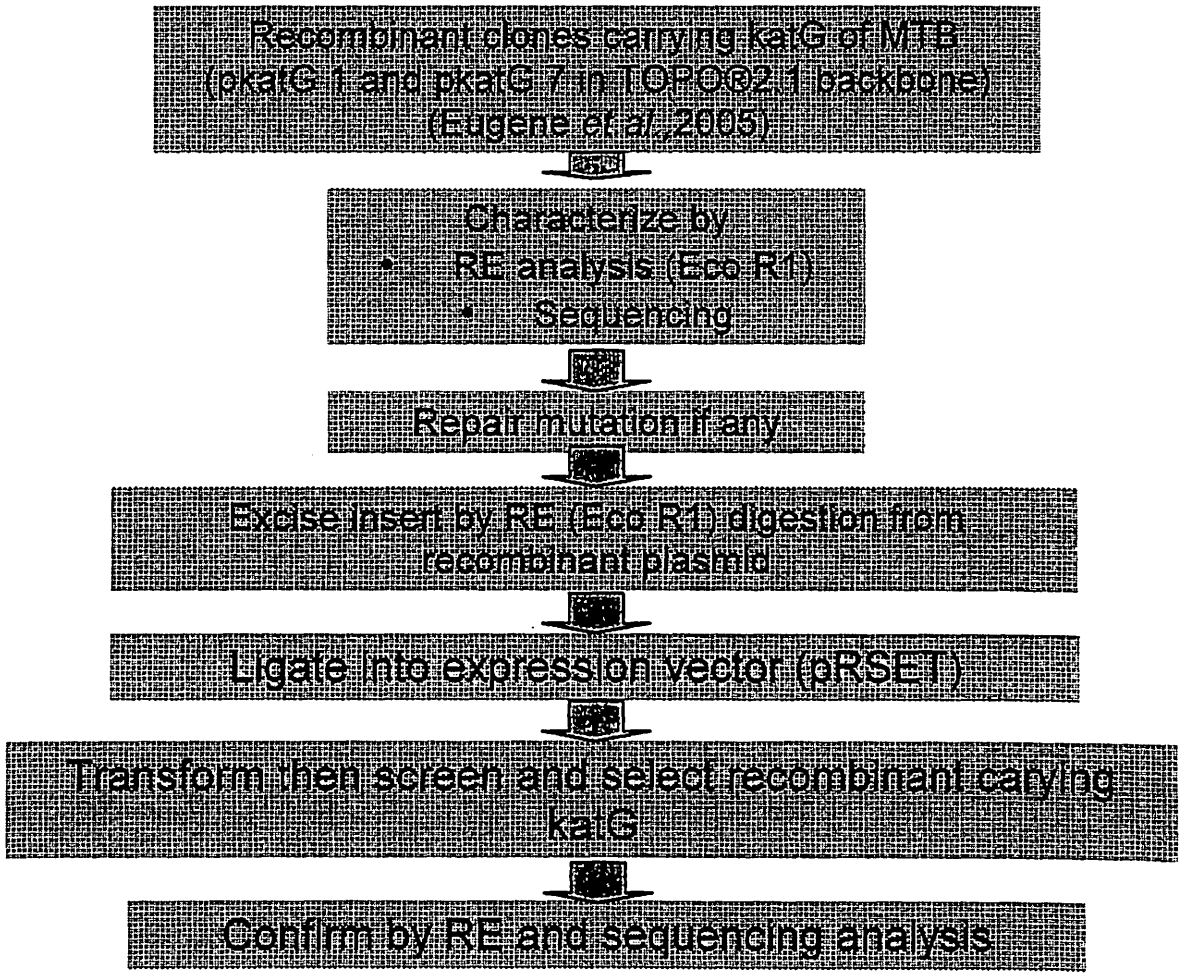

Figure 1.2: Flow chart of the study

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial Strains, culture and growth conditions

One strain of *Escherichia coli* was employed in this study, which is listed in the Table 2.1. *E. coli* DH5α has been used to propagate and maintain of KatG1 and KatG7 (in pRSET B). Liquid and solid agar plate cultures were prepared according to the standard methods as published (Sambrook *et.al*). Bacteria were grown in the Luria-Bertani (LB) medium. The culture was maintained at 37°C. Cells were maintained for longer storage in the form of glycerol stocks and stored in -80°C.

Table 2.1: Bacterial strain and its genotype used in this study

Bacteria species and strains	Genotype	Supplier
E.coli DH5α™	F- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR* *recA1* *endA1* *hsdR17*(rk-, mk+) *phoA*sup*E44* *thi-1* *gyrA96* *relA1* λ-	Invitrogen, USA

2.1.2 Plasmid

There are two clones that have been specifically amplified by using PCR technique, which are known as KatG1 and KatG7. The amplified target has been cloned into pCR®2.1-TOPO plasmid vector and maintained in *E.coli* DH5α glycerol stock. The plasmid was designated as pKatG1 and pKatG7. These glycerol stocks were obtained from previous study (Eygene *et.al.*,2005). The size of the gene is 2252 base pair in size.

2.1.3 Expression vector

The expression vector that has been used in this study is pRSET B. The map of vector is shown in Figure 2.1.

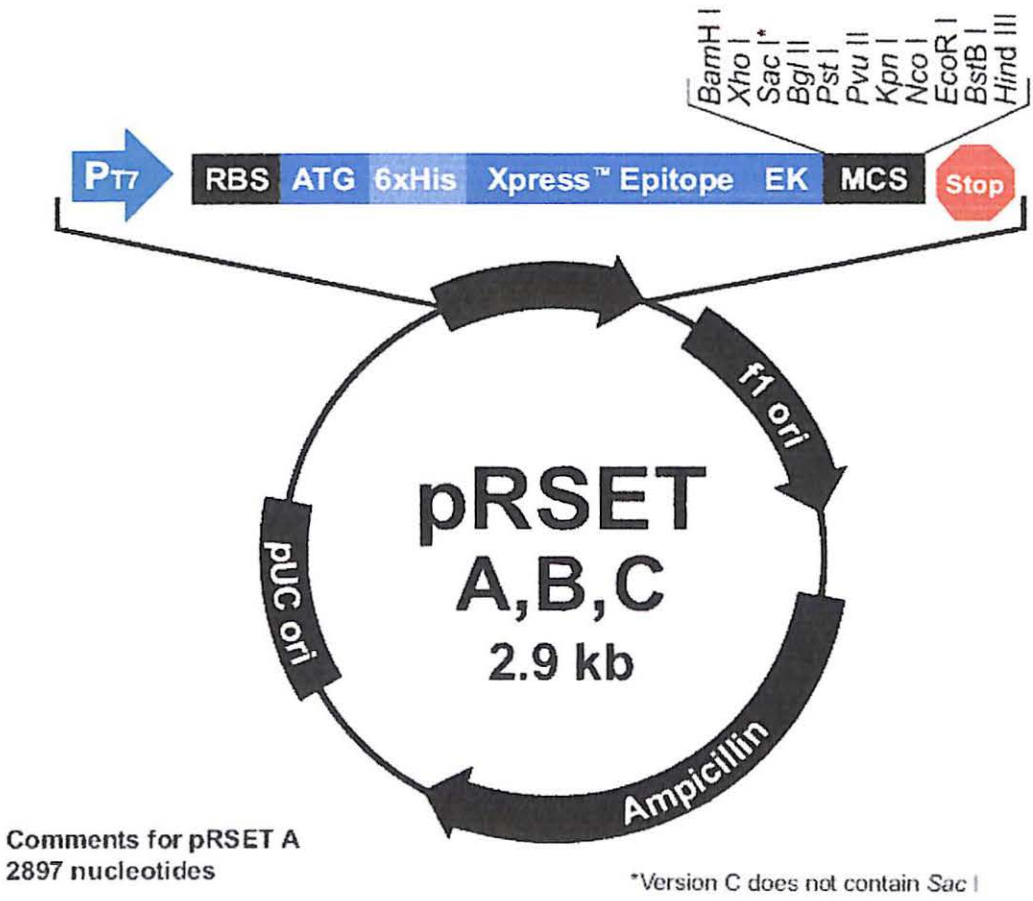

Figure 2.1: Map of pRSET A, B, and C.
(Adapted from https://www.invitrogen.com/content/sfs/manuals/prset_man.pdf)