STUDY OF TRANSPOSON LOCALIZATION ON GENOME OF MACROLIDE-SUSCEPTIBLE *MYCOBACTERIUM TUBERCULOSIS* H37RV MUTANTS

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การศึกษาตำแหน่งของทรานสโปซอนบนจีโนมของเชื้อ *MYCOBACTERIUM TUBERCULOSIS* H37RV สายพันธุ์กลายที่ไวต่อยากลุ่มแมโครลายด์

STUDY OF TRANSPOSON LOCALIZATION ON GENOME OF MACROLIDE-SUSCEPTIBLE *MYCOBACTERIUM TUBERCULOSIS* H37Rv MUTANTS

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ABSTRACT

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is still public health problem worldwide. Emergence of MDR-TB (Multidrug-Resistant Tuberculosis) and XDR-TB (Extensively Drug-Resistant Tuberculosis) have worsened the TB problem. Therefore, many efforts have focused on development of new anti-TB drugs. Study of resistance mechanism in *M. tuberculosis* could serve as a basic knowledge for understanding drug action and how bacteria become resistance and could be useful for development of new drugs. Alternatively, the drugs that have already used in human for treatment of other diseases and later found to have an anti-TB activity might be useful as anti-TB drugs. Clarithromycin (CLR) is a macrolide antibiotic used for treatment of mycobacterial diseases except tuberculosis because *M. tuberculosis* is naturally resistant to this drug. However, CLR-susceptible *M. tuberculosis* H37Rv mutants could be constructed by random transposon mutagenesis. In the present study, two CLR-susceptible *M. tuberculosis* H37Rv mutants, Tn-77 and Tn-196, were characterized for their transposon insertion sites and their genes associated with CLR-susceptible phenotype. The Tn*5* transposon DNA fragment was localized by genome walking. Results revealed that Rv0470A and overlapping region of *rpfB-ksgA* were disrupted by the transposon DNA fragment for Tn-77 and Tn-196, respectively. Rv0470A encodes a hypothetical protein whereas *ksgA* codes for dimethyladenosine transferase. Overexpression of each gene in *M. smegmatis* mc^2 155 increased Minimal Inhibitory Concentration (MIC) of CLR when compared to that of the parental strain, indicating the association of these genes with mycobacterial CLR resistance mechanism.

หัวข้อวิทยานิพนธ์	การศึกษาตำแหน่งของทรานสโปซอนบนจีโนมของเชื้อ *Mycobacterium tuberculosis* H37Rv สายพันธุ์กลายที่ไวต่อยากลุ่มแมโครลายด์
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บทคัดย่อ

วัณโรคเป็นโรคติดเชื้อที่เกิดจากเชื้อ *Mycobacterium tuberculosis* ซึ่งยังคงเป็นปัญหาทางสาธารณสุขทั่วโลก การรายงานพบเชื้อวัณโรคดื้อยาหลายขนาน (Multidrug-Resistant Tuberculosis, MDR-TB) และเชื้อวัณโรคดื้อยาเกือบทุกขนาน (Extensively Drug-Resistant Tuberculosis, XDR-TB) ทำให้ปัญหาวัณโรคทวีความรุนแรงยิ่งขึ้น ดังนั้นจึงมีความพยายามในการแก้ปัญหาวัณโรคหลายด้านซึ่งรวมถึงการค้นหาและพัฒนายาต้านวัณโรคชนิดใหม่ หรืออีกทางหนึ่งคือการนำยาที่ใช้รักษาโรคอื่นในคน ที่ต่อมาพบว่ามีฤทธิ์ในการฆ่าเชื้อวัณโรคมาประยุกต์ใช้ เช่น ยาคลาริโทรมัยซินซึ่งเป็นหนึ่งในยากลุ่มแมโครลายด์ที่ใช้รักษาโรคติดเชื้อมัยโคแบคทีเรียอื่น ที่ไม่ใช่วัณโรคเพราะเชื้อวัณโรคปกติดื้อต่อยานี้โดยธรรมชาติ จากการสร้างและทดสอบเชื้อวัณโรคกลายพันธุ์จากเชื้อวัณโรคสายพันธุ์ H37Rv ด้วยวิธี Transposon mutagenesis พบเชื้อสายพันธุ์กลายที่ไวต่อยาคลาริโทรมัยซิน จึงได้คัดเลือกมาศึกษาต่อในที่นี้ 2 สายพันธุ์ได้แก่ Tn-77 และ Tn-196 เพื่อหาตำแหน่งการแทรกของชิ้นส่วน Transposon ในสายพันธุ์ดังกล่าว ด้วยเทคนิค Genome walking และศึกษาความเกี่ยวข้องของยีนที่ถูกแทรกกับการไวต่อยาคลาริโทรมัยซิน ผลการทดลองพบว่าชิ้นส่วน Transposon เข้าไปแทรกที่ยีน Rv0470A และส่วนทับซ้อน (Overlapping) ของยีน *rpfB-ksgA* ของเชื้อสายพันธุ์กลาย Tn-77 และ Tn-196 ตามลำดับ ซึ่งยีน Rv0470A ถอดและแปลรหัสเป็นโปรตีนที่ยังไม่ทราบหน้าที่ ในขณะที่ยีน *ksgA* ถอดและแปลรหัสเป็นเอนไซม์ไดเมทิลอะดีโนซีนทรานสเฟอเรส นอกจากนี้ยังพบว่า หากทำให้ยีน Rv0470A หรือ *ksgA* (Rv1010) มีการแสดงออกเพิ่มมากขึ้น (overexpression) ในเชื้อ *M. smegmatis* mc^2 155 จะส่งผลให้เชื้อดังกล่าวมีค่าความไวต่อยา (MIC) เพิ่มมากขึ้นเมื่อเทียบกับค่าของเชื้อสายพันธุ์ดั้งเดิม แสดงให้เห็นว่ายีนทั้งสองนี้มีส่วนเกี่ยวข้องกับการดื้อยาคลาริโทรมัยซิน

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Rungaroon Suksamran

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CHAPTER 1

INTRODUCTION

1.1 Statement and significance of problem

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is the second most common infectious cause of adult mortality. TB is ranked 10th of all cause to loss of healthy life worldwide (Cole, 2005). Despite the declaration of TB as a global emergency by the World Health Organization 10 years ago, TB problem has worsened mostly due to increase in human immunodeficiency virus (HIV)-infected people, resulting in increasing incidence of TB and emergence of drug-resistant strains. Over 98% of deaths from TB appear in developing countries with high fatality rates in HIV-related TB patients. Not only the institutional outbreaks of multidrug-resistant TB (MDR-TB) were occurred but also have a report about occurring of extensively drug resistant tuberculosis (XDR-TB).

Resistance to anti-TB drugs can occur when these drugs are misused or mismanaged. Examples include when patients do not complete their full course of treatment, when health-care providers prescribe the wrong treatment, the wrong dose, or length of time for taking the drugs, when the supply of drugs is always not available or when the drugs are of poor quality. MDR-TB is defined as *M. tuberculosis* strain resistant to at least two of the most effective antituberculous drugs, isoniazid and rifampicin (Zager and McNerney, 2008). XDR-TB is a relatively rare type of MDR-TB. XDR-TB is defined as MDR-TB plus resistant to any fluoroquinolones and at least one of three injectable second-line drugs such as amikacin, kanamycin, or capreomycin. Because XDR-TB is resistant to first-line and second-line drugs, patients are left with less effective treatment options, and cases often have worse treatment outcomes. Persons with HIV infection or other conditions that can compromise the immune system are at highest risk for MDR-TB and XDR-TB (Singh *et al.*, 2007). They are more likely to develop TB disease once infected and have a higher risk of death from disease. Therefore international attention had refocused on TB and it became apparent that renewed efforts were required worldwide.

Many efforts aim to develop new anti-TB drugs, particularly for use against drug-resistant strains. Researches involved in screening anti-TB activity compounds and identifying potential drug targets for development of new drugs have been paid more attention. Alternatively, the drugs that have already used in human for treatment of other diseases and later found to have an anti-TB

activity might have been useful as anti-TB drug, particularly for the use against drug-resistant TB. Clarithromycin (CLR), a 14-membered macrolide drug, is potent against mycobacterial infection except tuberculosis. *M. tuberculosis* complex except some strains of *M. bovis* BCG are naturally resistant to macrolides, although they do not contain any mutations in the drug-binding site, the peptidyl transferase loop. Recent studies revealed that the methyltransferase (ErmMT) enzyme encoded by Rv1988 or *erm*(37) gene homolog in *M. tuberculosis* (Buriánková *et al.*, 2004 ; Madsen *et al.*, 2005 ; Andini and Nash, 2006) and a putative transcriptional activator, *whiB7*, are involved in the intrinsic macrolide resistance in *M. tuberculosis* complex (Morris *et al.*, 2005). However, there have been some clinical isolates of *M. tuberculosis* that showed clarithromycin-susceptible phenotype and did not contain any defect in known resistant genes, indicating other unknown resistance mechanisms. This warranted further investigation and may be helpful to the development of new anti-TB drugs.

Many methods have been used to construct *M. tuberculosis* mutants. In general, mutants have been constructed by several methods either randomly or specifically. Transposon mutagenesis is one of the promising methods that can generate mutants nonspecifically but the specific mutants can be selected upon the desired phenotype. In the present study, 2 clarithromycin-susceptible *M. tuberculosis* H37Rv mutants Tn-77 and Tn-196 constructed by transposon mutagenesis were investigated for their genes that are associated with clarithromycin susceptibility. Knowledge gained from this study would help to understand more about the mechanisms conferring intrinsic macrolide resistance in *M. tuberculosis* and would be valuable for use in development of new anti-TB drugs. In addition, it could provide a new alternative way for development of macrolides for use in treatment of TB.

1.2 Goal and objectives

This research aims to (i) localize the position of transposon DNA fragment integrated in genome of clarithromycin-susceptible *M. tuberculosis* H37Rv mutants, (ii) to identify the disrupted gene(s), and (iii) to investigate its role in clarithromycin resistance.

1.3 Scope of the study

1. Localization of transposon DNA fragement was achieved using Genome Walking Technique to extend a transposon sequence region to its uncharacterized flanking regions and

polymerase chain reaction (PCR) product was ligated into the TA cloning vector (pDrive, Qiagen, Germany), transformed into *E. coli* DH5α. DNA sequencing was performed for identifying the position of transposon.

2. A candidate gene was amplified by using PCR technique. A recombinant plasmid was constructed by subcloning the amplified gene into the TA cloning vector and transforming into *E. coli* DH5α. Transformants were analyzed for the presence of cloned gene. DNA sequencing and analysis were performed for determining the correct nucleotide sequence.

3. A replicative mycobacterial shuttle vector, pMV261, containing a candidate gene was constructed and transformed into *M. smegmatis* mc^2155 to generate the *M. smegmatis* mc^2155 strain carrying the candidate *M. tuberculosis* H37Rv gene.

4. Overexpression of the candidate gene in *M. smegmatis* mc^2155 was performed and investigated whether it plays a role in clarithromycin susceptibility.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is a common and often deadly infectious disease caused by members of *Mycobacterium tuberculosis* complex, mostly by *M. tuberculosis*. TB usually causes pulmonary disease but can also disseminate to other parts of the body. The bacteria spread through the air, when people who have the disease cough, sneeze, or spit. Most infections in humans result in an asymptomatic, latent infection, and about one in ten latent infections eventually progresses to active disease, which, if left untreated, kills more than half of its victims.

From WHO report in 2009, it was found that 9.4 millions incident cases of TB, 11.1 millions prevalent cases of TB, 1.3 millions deaths from TB among HIV-negative people and an additional 0.52 millions TB deaths among HIV-positive people were estimated. It is projected that TB will remain among the ten leading cause of global disease burden even in the year 2020. Thailand is ranked 18th of high TB burden countries, that have a lot of TB patients. There are 91,000 TB patients for annually and over 41,000 being infectious. Tuberculosis is still the second-rank of death due to infection disease and is the major cause of death among AIDS patients; 0.7% of HIV infection is in pregnant women.

Generally, TB can be cured with an effective antituberculous drugs but effective TB-treatment has been obstructed by a long time of treatment, resulting in intermittent treatment and leading to develop multidrug-resistant tuberculosis (MDR-TB). More seriously, there has been a report about extensively drug resistant Tuberculosis (XDR-TB), which not only has property like MDR-TB but also resist to second-line drugs and showed high mortality rate. Approximately 98% of XDR-TB patients died within 16 days after diagnosis (CDC, 2006).

As mentioned earlier, many countries had refocused on TB researches in several aspects, including pathogenesis, immunology, latency, vaccine and drug resistance. More understanding the pathogen will provide valuable information which can use for fighting against TB, particularly drug-resistant TB.

2.2 Mycobacteria

Mycobacteria are classified in kingdom Bacteria, class Actinomycetes, family Mycobacteriaceae and genus *Mycobacterium*. This genus includes pathogens known to cause serious diseases in mammals, including tuberculosis and leprosy. Mycobacteria are aerobic and nonmotile bacteria with 2-4 μm in length and 0.2-0.5 μm in width (Fig. 2.1) and are characteristically acid-fastness. Mycobacteria do not contain endospores or capsules, and are usually considered as Gram-positive bacteria. All species share a characteristic cell wall that is thicker than that of many other bacteria. A natural division occurs between slowly and rapidly growing species. Mycobacteria that form colonies clearly visible to the naked eye within 7 days on primary culture are termed rapid growers, while those requiring longer than 7 days are termed slow growers. Based on colony morphology, growth rate and pigment production mycobacteria can be classified into non-culturable mycobacteria like *M. leprae*, nontuberculous mycobacteria or NTM and *M. tuberculosis* complex or MTC that causes TB both in human and animals.

Figure 2.1 *M. tuberculosis* scanning electron micrograph
Available : http://feww.files.wordpress.com/2008/10/tb.jpg

2.2.1 Mycobacterial Cell Wall Structure

The cell wall structure of mycobacteria contains peptidoglycan, but otherwise it is composed of complex lipids (Fig. 2.2). The cytoplasmic membrane is encapsulated by a layer of peptidoglycan. The peptidoglycan backbone is attached to arabinogalactan through an unusal disaccharide phosphate linker region. The arabinogalactan is a branched-chain polysaccharide consisting of a proximal galactose chain linked to a distal arabinose chain. The hexaarabinofuranosyl termini of arabinogalactan are esterified to mycolic acids. The mycolic acid

chains are shown perpendicular to the cytoplasmic membrane with the exposed chains interacting with the mycolic chains of trehalose dimycolate. Another major component non-covalently associated to the mycobacterial cell wall is the immunogenic lipoarabinomannan, which is attached to the cytoplasmic membrane by a phosphatidylinositol anchor. Small and hydrophilic solutes diffuse through water-filled protein channels, porins, whereas hydrophobic compounds use the lipid pathway. Proteins are represented by solid oval bodies. The lipid fraction of mycobacterial cell wall consists of three major components, mycolic acids, cord factor, and wax-D. Mycolic acids are unique alpha-branched lipids found in cell walls of Mycobacterium and Corynebacterium. They make up 50% of the dry weight of the mycobacterial cell envelope. The cell wall structure of mycobacteria is a major determinant of virulence for the bacterium.

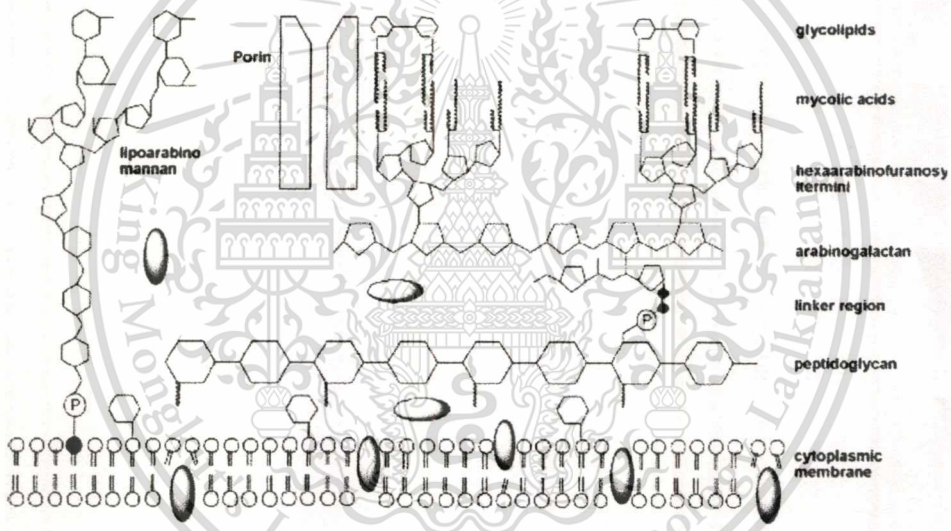


Figure 2.2 The cell wall structure of mycobacteria

Available : http://www.scielo.br/img/revistas/mioc/v100n6/a01fig01.gif

2.2.2 Virulence

Virulence is a mycobactericidal ability to live and divide in macrophage (Barry *et al.*, 1998). Mycobacteria do not possess the classic bacterial virulence factors such as toxins or capsules. However, a number of structural and physiological properties of these bacteria are beginning to be recognized for their contribution to bacterial virulence and the pathology of tuberculosis, because of three major components, mycolic acids, cord factor, and wax-D.

Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic acids are thought to be a significant determinant of virulence in mycobacteria. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum.

Cord Factor is responsible for the serpentine cording and toxic to mammalian cells. Cord factor is most abundantly produced in virulent strains of mycobacteria. This component has involved in properties of the bacterium for impermeability to stains and dyes, resistance to many antibiotics, killing by acidic and alkaline compounds, osmotic lysis via complement deposition, lethal oxidations and survival inside of macrophages.

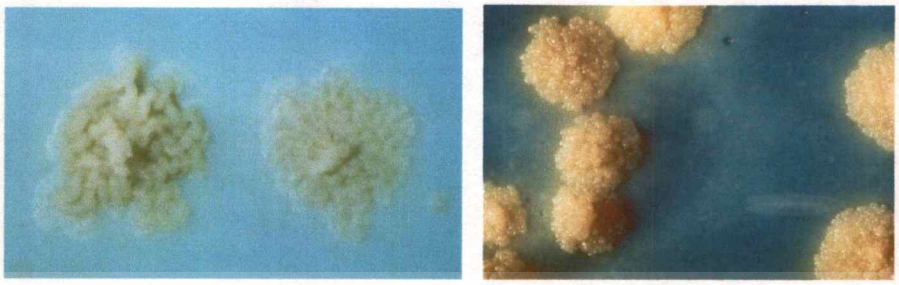
Mycobacterial wax D and peptidoglycans have all been found to be arthritogenic. Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA), an antigen solution emulsified in mineral oil, used as an immunopotentiator. It is effective in stimulating cell-mediated immunity and may lead to the potentiation of the production of certain immunoglobulins.

2.3 *Mycobacterium tuberculosis*

M. tuberculosis (MTB) was first identified in 1882 by Robert Koch, who was awarded the Nobel Prize in Physiology or Medicine for these tuberculosis findings in 1905. MTB is a member of *M. tuberculosis* complex (MTC), consisting of *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium canetti*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *M. tuberculosis* and is a causative agent of tuberculosis, mostly in human. This pathogen is known for a long time as "Tubercle bacillus" or "Koch's bacilli" (Camus *et al.*, 2002). MTB is an obligate aerobic bacterium. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs. In macrophage, it is a facultative intracellular parasite and has a slow generation time, 15-20 hr, a physiological characteristic that may contribute to its virulence.

MTB is grown in basic component medium but prefer fat media. Optimal condition for growth is 38 °C and pH 6.6. Two media are used to grow MTB; Middlebrook's medium which is a chemical based medium and Lowenstein-Jensen medium which is an egg based medium. The colonies of MTB are small and buff colored when grown on either medium. Both types of media

contain inhibitors to keep contaminants from out-growing organisms. It takes 4-6 weeks to get visual colonies on either type of media (Fig. 2.3).



(A) (B)

Figure 2.3 Colonies of *M. tuberculosis* on Lowenstein-Jensen medium (A) and on Middlebrook 7H10 agar medium (B)

Available:http://staff.vbi.vt.edu/pathport/pathinfo_images/Mycobacterium_tuberculosis/TBLabwork_TBonMedia.jpg

Available: http://upload.wikimedia.org/wikipedia/commons/0/0a/TB_Culture.jpg

Mycobacterium species are classified as acid-fast bacteria due to their cell wall mycolic acid. One of acid-fast staining methods for *M. tuberculosis* is the Ziehl-Neelsen staining. When this method is used, MTB is stained with carbol-fuchsin (a pink dye) and is resistant to decolorize with acid-alcohol. After the smear is counterstained with methylene blue, acid-fast bacilli appear pink in a contrasting background (Fig. 2.4).

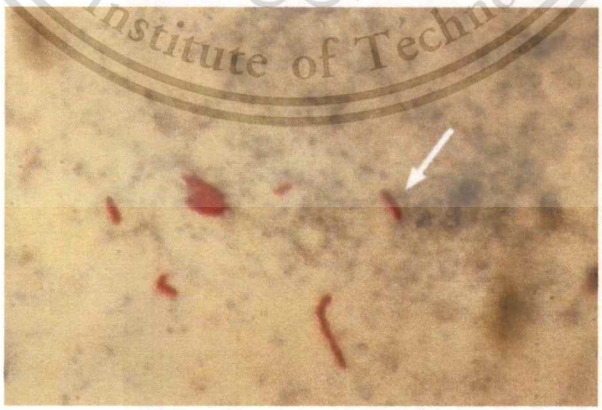


Figure 2.4 Acid-fast staining of *M. tuberculosis* by Ziehl-Neelsen method

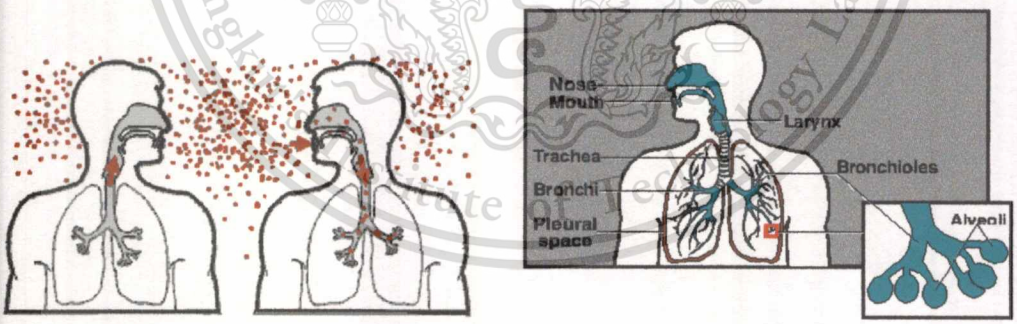
Available : http://depts.washington.edu/hivaids/oit/case5/fig8d.html

2.4 Immunology and pathogenesis

TB infection means that MTB is in the body, but the immune system is keeping the bacteria under control. The immune system does this by producing macrophages that surround the tubercle bacilli. The cells form a hard shell that keeps the bacilli inside and under control. Most people with TB infection have a positive reaction to the tuberculin skin test. People, who have TB infection but not TB disease, are not infectious; they cannot spread the infection to other people. These people usually have a normal chest x-ray. TB infection is not considered a case of TB disease.

Only 3-4% of infected individuals will develop active disease upon initial infection, 5-10% within one year. These percentages are much higher if the individual has HIV positive. The following stages that will be explained are for a MTB - sensitive host. It should be realized that, only a small percent of MTB infections progress to disease and even a smaller percent progress all the way to be transmittable. Usually the host will control the infection at some point.

Disease progression starts when droplet nuclei are inhaled. One droplet nuclei contains no more than 3 bacilli and is generated by during talking, coughing and sneezing (Fig. 2.5 A) and they can remain air-borne for extended periods of time. Droplet nuclei may reach the small air sacs of the lung (the alveoli) (Fig. 2.5 B), where infection begins.



(A) (B)

Figure 2.5 Spread of droplet nuclei from one individual to another (A) and tuberculosis begins when droplet nuclei reach the alveoli (B)

Available: http://textbookofbacteriology.net/themicrobialworld/lungsandalveoli.gif

Available: http://web.njcu.edu/dept/hwc/Uploads/aerosoltransmission.jpg

After initial infection (Fig. 2.6), lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented MTB antigen in context of Major Histcompatibility Complex (MHC) molecules. This results in T-cell activation and the liberation of cytokines including gamma interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying MTB (Houben *et al.*, 2006) (Fig. 2.7). It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an MTB infection. An antibody mediated immune (AMI) will not aid in the control of a MTB infection because MTB is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in its cell wall. Although a CMI response is necessary to control an MTB infection, it is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-l), tumor necrosis factor (TNF), and gamma IFN. It is also at this stage that tubercle formation begins. The center of the tubercle is characterized by "caseation necrosis" (Kim *et al.*, 2003), meaning it takes on a semi-solid or "cheesy" consistency. MTB cannot multiply within these tubercles because of the low pH and anoxic environment. MTB can, however, persist within these tubercles for extended periods.

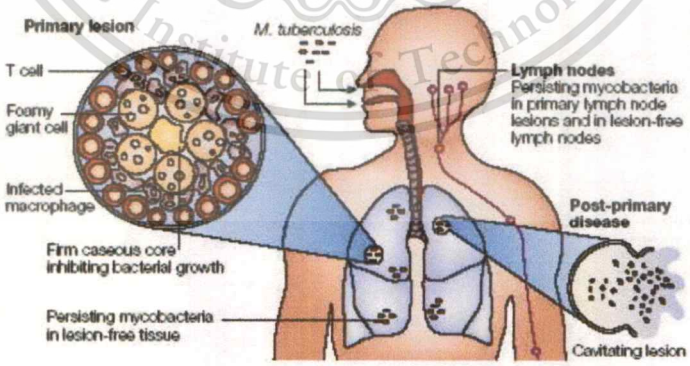


Figure 2.6 Tuberculosis infection

Available: http://staff.vbi.vt.edu/pathport/pathinfo_images/Mycobacterium_tuberculosis TBInfection_Fig1_StewartPersistentTB.jpg

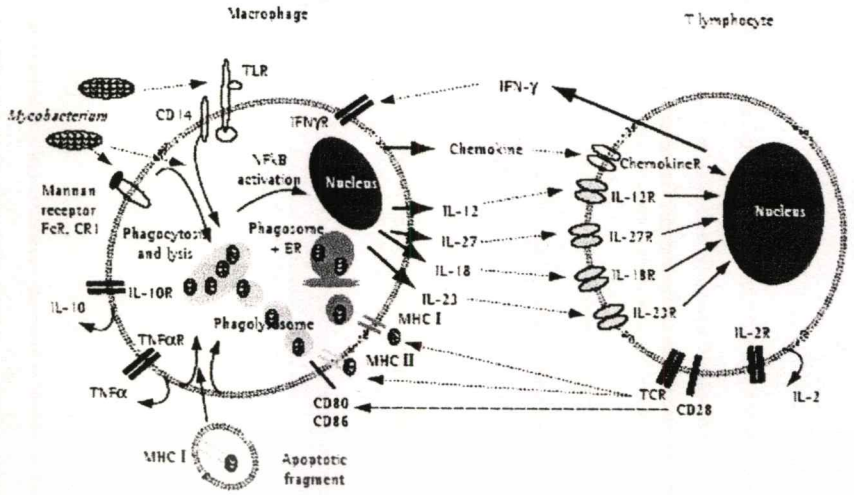


Figure 2.7 Mechanisms involved in the activation of macrophages and T lymphocytes by *M. tuberculosis*
Source : Teixeira *et al* (2007)

Although many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. MTB uses these macrophages to replicate, and hence, the tubercle grows. The growing tubercle may invade a bronchus. If this happens, MTB infection can spread to other parts of the lung. Similarly the tubercle may invade an artery or other blood supply line. The hematogenous spread of MTB may result in extrapulmonary tuberculosis otherwise known as milliary tuberculosis. The secondary lesions caused by miliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes and peritoneum. The caseous center of the tubercle is very conducive to MTB growth and the organism begins to rapidly multiply extracellularly. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation and also allows MTB to spill into other airways and rapidly spread to other parts of the lung.

2.5 Symptoms

Over 75% of the patients where TB becomes an active disease affect the lungs called pulmonary TB. Symptoms include prolonged cough of more than three weeks duration, chest pain and coughing up blood. Systematic symptoms include fever, chills, night sweats, appetite loss, weight loss and paling, and those afflicted are often easily fatigued. When the infection spreads out of lungs, extrapulmonary TB occurs, including TB of central nervous system, lymphatic

system, and geniturinary system. A serious form of TB is disseminated or miliary tuberculosis. Extrapulmonary TB is common in immunosuppressed persons and in young children.

2.6 Diagnosis of tuberculosis

Primary diagnosis of tuberculosis still relies on acid-fast staining and microscopic examination. This method is easily to perform but lacks of sensitivity (need $>10^4$ cells/ml) and specificity. Definite diagnosis of tuberculosis is to isolate *M. tuberculosis* directly from clinical samples. Conventional culture of tubercle bacilli has been performed using Löwenstein-Jensen medium and requires 6–8 weeks until colonies appear. However, a rapid culture method using automated culture system has also been used to shorter time to 7-10 days for pathogen detection. Moreover, both methods require specie identification, which takes additionally 4-6 weeks, if biochemical identification is used. Identification time can be shorter to 1-2 working days, when the genetic methods are used (Cole, 2005)

Alternatively, polymerase chain reaction (PCR) or other molecular techniques (Cole, 2005) have been used as diagnostic tool to detect the presence of *M. tubercuosis* genetic material, mostly DNA, directly from clinical samples. These methods showed promising result with high sensitivity and specificity, particularly when tested with smear-positive samples. PCR is a relatively new development in active TB testing. Even though PCR techniques can magnify even the smallest amounts of genetic material, the sample used still has to contain a certain number of TB bacteria and this is not always possible, particularly with non-pulmonary TB where sensitivity of the test is low, the laboratory will often culture the sample, to allow the bacteria to multiply, before carrying out the PCR test; this can take several days or weeks. The test is also relatively complicated to run in the laboratory because it is prone to cross contamination and relatively expensive.

2.7 Chemotherapy of tuberculosis and drug-resistant mechanisms

Chemotherapy of tuberculosis was succeeded by using antituberculous drugs. Drugs used to treat tuberculosis are classified into the first-line and the second-line drugs. The first-line essential anti-tuberculosis drugs are the most effective, and are a necessary component of any short-course therapeutic regimen. The drugs in this category are isoniazid, rifampin, ethambutol, pyrazinamide and streptomycin. The mechanism of some of these drugs is shown in Fig. 2.8. However, these drugs

have been introduced nearly 30 years and are likely ineffective for treatment of drug resistant strain, leading to use either more expensive, more toxic, and/or less effective second-line drugs as therapeutic agents (Mitnick *et al.*, 2003). The second-line anti-tuberculosis drugs are clinically much less effective than the first-line agents and elicit severe reactions much more frequently. These drugs include ethionamide, fluoroquinolone, cycloserine, para-aminosalicylic acid (PAS), amikacin, kanamycin and capreomycin.

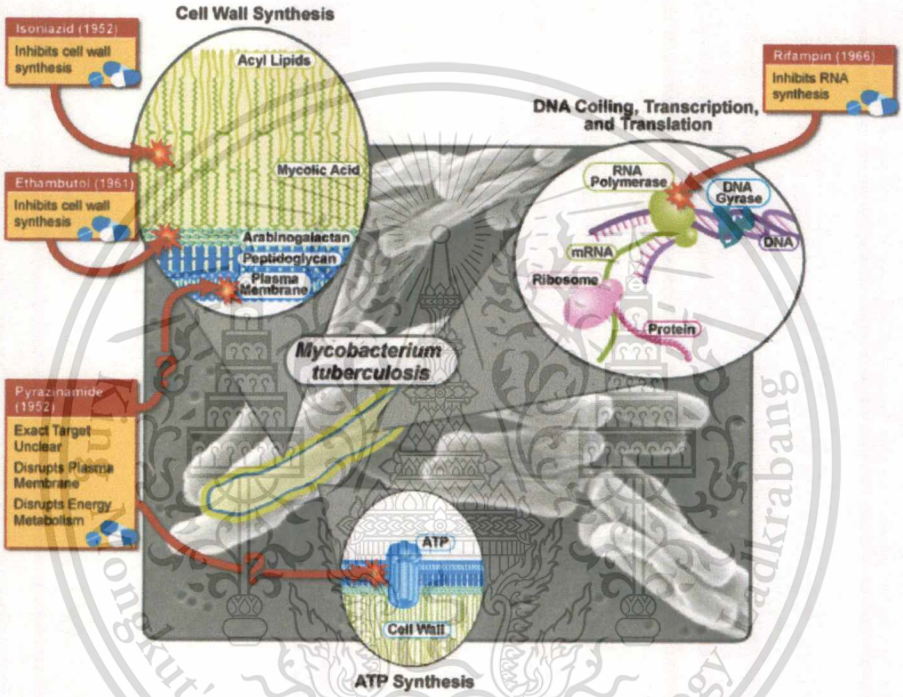


Figure 2.8 Various pharmaceutical tuberculosis treatments and their action Available:http://upload.wikimedia.org/wikipedia/commons/0/08/Tuberculosis-drugs-and-actions.jpg

However, the emergence of MDR-TB and XDR-TB was a major cause of antituberculous drug failure because of the drug-resistant mechanisms of these strains. Because of the difference in mechanism action of each drug, Cole (2005) explained that bacteria can become resistant to antibiotics or antibacterial agents by a number of common strategies, including target modification, target overexpression, barrier mechanism, drug-inactivating enzymes, inactivation

of drug-activating enzymes, and drug extrusion mechanisms. The mechanisms of antituberculous drug action and resistance mechanisms were described below.

Isoniazid (INH) was first discovered as an antituberculous drug in 1951 (Rattan *et al.*, 1998; Somoskovi *et al.*, 2001). The drug was activated by a catalase- peroxidase encoded by *katG* to be an active form (isonicotinic acid), which is a toxic substance in the bacterial cell (Zhang *et al.*, 1992). The activated INH subsequently inhibits mycolic acid biosynthesis, which is a major cell wall component. One of the drug targets is InhA, an enoyl-acyl carrier protein reductase encoded by *inhA*. Inhibition of InhA results in mycolic acid biosynthesis and ultimately leads to cell lysis (Cole, 2005). Mutations in *inhA* have been found to confer INH resistant due to the decreased binding affinity of InhA to NADH, which can protect cell from activated INH (Johnson *et al.*, 2009). Another mechanism for INH resistance found in *M. smegmatis* was mutations in *ndh*, which encodes NADH dehydrogenase that is bound to the active site of InhA to form the ternary complex with activated INH (Miesel *et al.*, 1998). Approximately 70–80% of INH resistance in clinical isolates of *M. tuberculosis* can be attributed to mutations in the *kat*G and *inh*A genes (Ramaswamy and Musser, 1998). Several studies have suggested that low-level of INH resistance is correlated with resistance to ethionamide (ETH), a second line drug that has similar mode of action to INH (Johnson *et al.*, 2009).

Rifampicin (RIF) was first introduced in 1972 as an antituberculous drug (Rattan *et al.*, 1998; Ramaswamy and Musser, 1998) and is the most effective anti-TB drug. RIF binds to the ß-subunit of DNA-dependent RNA polymerase, resulting in inhibition of transcriptional initiation (McClure and Cech, 1978) and thereby killing the organism. Mutations in ß-subunit of RNA polymerase encoding gene, *rpoB*, either short insertion, deletion or point mutation lead to RIF resistance. Studies on *rpoB* in RIF-resistant isolates of *M. tuberculosis* identified a variety of mutations and short deletions within the gene. More than 95% of all missense mutations are located in a 51-bp core region (Rifampicin resistance determining region or hot-spot region) of the *rpoB* between codons 507–533, with the most common changes at Ser531Leu, His526Tyr and Asp516Val. These amino acid substitutions occur in more than 70% of RIF-resistant isolates (Rattan *et al.*, 1998; Ramaswamy and Musser, 1998). Furthermore, the minimal inhibitory concentration (MIC) showed that the high level of RIF resistance is associated with mutations in codons 526 and 531, whereas alterations in codons 511, 516, 518 and 522 result in low level RIF resistance.

Johnson and coworkers (2009) explained that pyrazinamide (PZA), a nicotinamide analog, was first discovered to have an antituberculous activity in 1952. PZA has no effect on other mycobacteria but highly specific for *M. tuberculosis*. PZA is active against semi-dormant *M. tuberculosis* under acidic conditions, where pyrazinoic acid (POA) accumulates in the cytoplasm due to an ineffective efflux pump. PZA was converted to its active form, POA by the pyrazinamidase (PZase) encoded by *pncA* (Cynamon *et al.*, 1992) and most point mutations occurred in this gene lead to PZA resistance. Mutations, either base substitution, insertion, or deletion, in the *pncA* gene result in lacking of pyrazinamidase activity, suggesting the inability of these resistant strains to convert PZA to pyrazinoic acid (Portugal *et al.*, 2004). Approximately 70% of PZA-resistant clinical isolates were caused by *pncA* mutation. However, observation of PZA resistant isolates without *pnc*A mutation was also found, suggesting that another mechanism may be involved in PZA resistance (Johnson *et al.*, 2009).

Ethambutol (EMB) is effective only to mycobacteria and always used in combination with other first-line drugs. EMB acts by inhibiting arabinosyl transferase (EmbB) activity, resulting in inhibition of arabinogalactan biosynthesis that is a component of mycobacterial cell wall (Takayama and Kilburn, 1989). Three genes, designated *embCAB*, have been identified and encoded homologous arabinosyl transferase (Telenti *et al.*, 1997). Mutations in the embCAB operon were identified in up to 65% of EMB resistant clinical isolates of *M. tuberculosis*. The most frequently mutation was found at codon 306 of *embB* (Ramaswamy *et al.*, 2000; Sreevatsan *et al.*, 1997; Telenti *et al.*, 1997), but mutations at amino acid residues Asp328, Gly406, and Glu497 were also reported (Ramaswamy *et al.*, 2000; Sreevatsan *et al.*, 1997). The codon 306 region is highly conserved among the various EMB proteins and among different mycobacteria (Alcaide *et al.*, 1997; Lety *et al.*, 1997; Telenti *et al.*, 1997).

Streptomycin (STR), an aminocyclitol glycoside, was the first discovered antituberculous drug. STR, however, is rarely used nowadays because it is an injected drug and causes severe side effects. STR inhibits protein synthesis by interacting with 16S rRNA (*rrs*) and S12 ribosomal protein (*rpsL*), resulting in inducing ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis (Finken *et al.*, 1993). Ramaswamy and Musser (1998) reported that approximately 65–67% of STR resistant isolates had point mutations in *rrs* and *rpsL*. In addition, Meier and colleagues (1996) suggested that a low level of STR resistance is also associated with the alteration of cell permeability or rare mutations, which lie outside of the *rrs* and *rpsL*. Other aminoglycosides, such as amikacin (AMK) and kamamycin (KM), are second-

line antituberculous drugs. AMK and KM bind to bacterial ribosomes and disturb the elongation of the peptide chain in the bacteria rendering protein synthesis inhibition. Alangaden and coworkers (1998) reported that a point mutation at *rrs* position 1400 (A⟶G) conferred high-level of KM in *M. tuberculosis*.

Fluoroquinolones (FQ), including ciprofloxacin ofloxacin, gatifloxacin, levofloxacin, moxifloxacin, and sparfloxacin, are second-line antituberculous drugs and currently used to treat multidrug-resistant tuberculosis. Drugs bind to DNA gyrase encoded by *gyrA* and *gyrB*. This enzyme is an ATP-dependent type II DNA topoisomerase that catalyses negative supercoiling of DNA; inhibition of the enzyme results in inhibition of DNA replication. Mutations in *gyrA* and *gyrB* genes have been found to be associated with FQ resistance and are mostly clustered in a short region of 40 amino acids of GyrA, designated as the quinolone resistance determining region (QRDR). Approximately 42-85% of FQ-resistant clinical isolates of *M. tuberculosis* were associated with *gyrA* mutation (Takiff *et al.*, 1994; Alangaden *et al.*, 1995; Sullivan *et al.*, 1995). In addition, missense mutations at codon 90 and 94 of GyrA have been reported to confer different level of FQ resistance (Xu *et al.*, 1996). However, other resistance mechanisms including mutations elsewhere in *gyrA* or in *gyrB*, decreased drug permeability and active drug efflux remain to be investigated.

2.8 Macrolide

Macrolides are a group of antibiotics whose activity stems from the presence of a macrolide ring, a large macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine, may be attached. The lactone rings are usually 14, 15 or 16 membered. Macrolides belong to the polyketide class of natural products. Natural macrolide such as erythromycin are not effective against mycobacteria, but semisynthetic derivatives such as clarithromycin and azithromycin, have stronger antimycobacterial activities and are widely used to treat infections caused by some nontuberculous mycobacteria (Dautzenberg *et al.*, 1991).

Macrolides are used to treat infections such as respiratory tract and soft tissue infections. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and therefore macrolides are a common substitute for patients with a penicillin allergy. Beta-hemolytic streptococci, pneumococci, staphylococci and enterococci are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against mycoplasma, mycobacteria, some rickettsia, and chlamydia. Macrolide antibiotics do so by binding reversibly to the 50S

subunit of the bacterial ribosome (Hansen *et al.*, 2002). This action is mainly bacteriostatic, but can also be bactericidal in high concentrations. Macrolides tend to accumulate within leukocytes, and are therefore actually transported into the site of infection.

2.8.1 Mechanism of macrolide action

Macrolides are protein synthesis inhibitor. The mechanism of action of macrolides is inhibition of bacterial protein biosynthesis (Fig. 2.9), and they are thought to do this by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid as well as inhibiting ribosomal translocation. Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome.

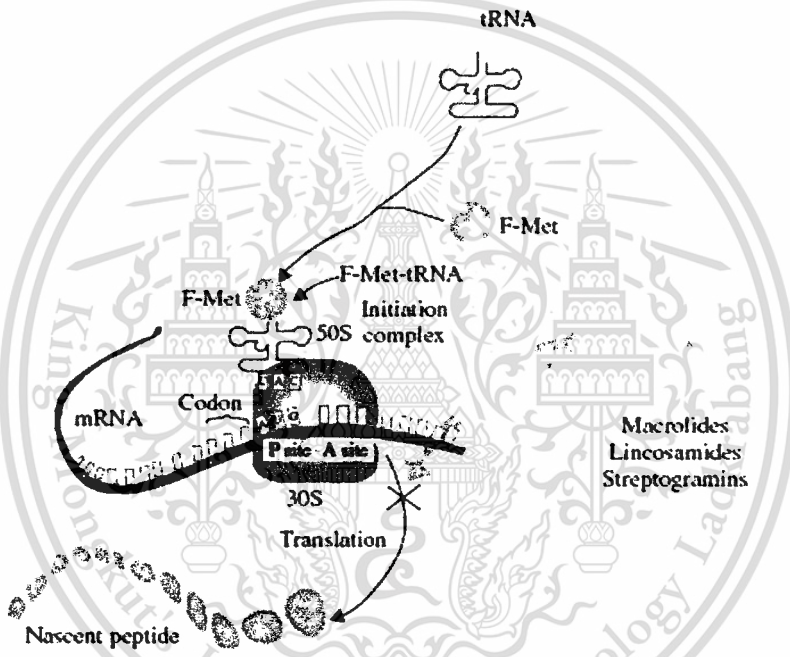


Figure 2.9 The mode of action of macrolides

Source : Tenson *et al* (2003)

2.8.2 Macrolide resistance

Mechanisms of macrolide resistance have been characterized for a broad range of gram-positive and gram-negative bacteria, including pathogenic isolates and macrolide producers. The primary mean of bacterial resistance to macrolides occurs by post-transcriptional methylation of the 23S bacterial ribosomal RNA. This acquired resistance can be either plasmid-mediated or chromosomal through mutations, and results in cross-resistance to macrolides, lincosamides, and streptogramins (an MLS-resistant phenotype) (Leclercq and Courvalin, 1991). Other macrolide

resistance mechanisms involve antibiotic inactivation, active drug efflux, and mutated ribosome components (ribosomal proteins or 23S rRNA) (Weisblum, 1998). In NTM, mutations in the 23S rRNA gene are only acquired macrolide resistance mechanism being characterized so far (Vester and Douthwaite, 2001). Since mycobacteria possess only one or two rRNA operons, mutations in one of them is sufficient to confer a resistance phenotype (Sander *et al.*, 1997). Macrolide resistance is frequently acquired during macrolide therapy due to 23S rRNA mutations (Meier *et al.*, 1994; Nash and Inderlied, 1995; Wallace *et al.*, 1996).

M. tuberculosis is intrinsically resistant to macrolide, even it contains similar drug-binding sequence like other macrolide-sensitive mycobacteria. Mechanism of resistance has been recently defined and found to be associated with methylation of the drug-binding target by methyltransferase (ErmMT) (Buriánková *et al.*, 2004) In addition, a putative transcriptional regulator WhiB7 has also been identified and involved in macrolide resistance (Morris *et al.*, 2005).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial strains

3.1.1 Mycobacterium tuberculosis H37Rv mutant strains Tn-77 and Tn-196 were obtained from Dr. Therdsak Prammananan, National Center for Genetic Engineering and Biotechnology, Thailand

3.1.2 *Mycobacterium smegmatis* mc^2155 (Snapper *et al.*, 1990) was obtained from Prof. Dr. Erik C. Böttger, Institute for Medical Microbiology, University of Zürich, Switzerland

3.1.3 *Escherichia coli* DH5α (F- φ80*lacZ*ΔM15 Δ (*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk^-, mk^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-) (Invitrogen, Carlsbad, USA)

3.2 Plasmids

3.2.1 pDrive Cloning Vector (Appendix A) (Qiagen, Hilden, Germany)

3.2.2 pMV261 (Stover *et al.*, 1991) was obtained from Prof. Dr. Erik C. Böttger (Appendix A)

3.3 Chemical reagents

3.3.1 Culture media

3.3.1.1 Luria-Bertani (LB) medium (Appendix B)

3.3.1.2 Middlebrook 7H10 medium (Appendix B) (Becton Dickinson, USA)

3.3.1.3 SOB medium (Appendix B)

3.3.1.4 OADC (Oleic acid-albumin-dextrose-catalase) (Becton Dickinson, USA) (Appendix B)

3.3.2 Antibiotics

3.3.2.1 Kanamycin (Sigma Aldrich, Steinheim, Germany)

3.3.2.2 Clarithromycin (Sandoz, Germany)

3.3.3 Enzymes

3.3.3.1 *Taq* DNA polymerase (Promega, Madison, USA)

3.3.3.2 Lysozyme (Amresco, Ohio, USA)

3.3.3.3 *Bam*HI (Biolabs, England)

3.3.3.4 *Eco*RI (Biolabs, England)

3.3.3.5 *Hin*dIII (Promega, Madison, USA)

3.3.3.6 T4 DNA ligase (Fermentas, Life Sciences, USA)

3.3.3.7 RNase A (Qiagen, Hilden, Germany)

3.3.4 DNA markers

3.3.4.1 λ DNA/*Hin*dIII fragments (Invitrogen, Carlsbad, USA)

3.3.4.2 100 bp Ladder DNA (Promega, Madison, USA)

3.3.5 Chemicals

3.3.5.1 Agarose (Research Organics, Ohio, USA)

3.3.5.2 Bacteriological agar (Scharlau, Barcelona, Spain)

3.3.5.3 Boric acid (Merck, Darmstadt, Germany)

3.3.5.4 Calcium chloride (Scharlau, Barcelona, Spain)

3.3.5.5 Chloroform-isoamyl-alcohol (Labscan, Dublin, Ireland)

3.3.5.6 CTAB (Cetyl Trimethyl Ammonium Bromide) (Sigma, St. Louise, USA)

3.3.5.7 dNTPs (Deoxynucleotide triphosphates) (Promega, Madison, USA)

3.3.5.8 EDTA (Ethylenediaminetetraacetic acid) (Bio Basic, Ontario, USA)

3.3.5.9 Ethanol (Fisher, Springfield, USA)

3.3.5.10 Gel star® (Cambrix Bio Science, Rockland, USA)

3.3.5.11 Glacial acetic acid (Labscan, Dublin, Ireland)

3.3.5.12 Glycerol (Fluka, Buchs, Schweizerland)

3.3.5.13 Hydrochloric acid (Labscan, Dublin, Ireland)

3.3.5.14 IPTG (Isopropyl- β - D-thiogalactopyranoside) (Bio Basic, Ontario, USA)

3.3.5.15 Manganese chloride (Scharlau, Barcelona, Spain)

3.3.5.16 MOPs (3-(N-morpholino)propanesulfonic acid) (Sigma, St. Louise, USA)

3.3.5.17 Peptone (Biomark™, Pune, India)

3.3.5.18 Potassium chloride (Scharlau, Barcelona, Spain)

3.3.5.19 Potassium acetate (Fisher, Springfield, USA)

3.3.5.20 Proteinase K (USB, USA)

3.3.5.21 Sodium chloride (Ajax, Finechem, New Zealand)

3.3.5.22 Sodium dodecyl sulfate (Promega, Madison, USA)

3.3.5.23 Sodium hydroxide (Labscan, Dublin, Ireland)

3.3.5.24 Tris (USB, Cleveland, USA)

3.3.5.25 Tween 80 (Fluka, Buchs, Schweizerland)

3.3.5.26 X-gal (5-Bromo-4-chloro-indoLyl- β -D-galactopyranoside) (Bio Basic, Ontario, USA)

3.3.5.27 Yeast extract (Himedia®, Mumbai, India)

3.3.6 Kits

3.3.6.1 QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany)

3.3.6.2 QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany)

3.3.6.3 QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany)

3.3.6.4 DNA Walking *SpeedUp*™ Premix Kit (Seegene, Korea)

3.3.6.5 QIAGEN PCR Cloning Kit (Qiagen, Hilden, Germany)

3.4 Instruments

3.4.1 Autoclave (Hiclave HV-50, Hirayama, Japan)

3.4.2 Balance (BD2215, Sartorius AG, Germany)

3.4.3 Digital dry bath (D1100 AccuBlock™, Labnet, USA)

3.4.4 DNA thermal cycler (DNA thermal cycler 480, Perkin Elmer, USA)

3.4.5 Documentation gel analysis (DBT-08, Syngene, Germany)

3.4.6 Electrophoresis equipments (GNA 100, Pharmacia Biotech, Sweden)

3.4.7 Glass Wares (Pyrex, Germany)

3.4.8 Incubator (D-78532, Binder control, Germany)

3.4.9 Incubator shaker (Innova 4000, New Brunswick Scientific, Germany)

3.4.10 Laminar air flow cabinet (HS123, International Scientific Supply, Thailand)

3.4.11 Microcentrifuge (Spectrafuge 16M, Labnet, USA)

3.4.12 Microtitreplate (U shape) (Thermo Scientific, USA)

3.4.13 Microplate reader (Labsystems iEMS Reader MF, Labsystems, Finland)

3.4.14 pH meter (Cyberscan 2000, Eutech Cybernetics, Singapore)

3.4.15 Power supply (EPS 301 Amersham pharmacia, Biotech, Sweden)

3.4.16 Refrigerated centrifuge (Falcon 6/300, Sanyo, Japan)

3.4.17 Vortex (Genie 2, Scientific Industries, USA)

3.5 Methods

3.5.1 Growth conditions

3.5.1.1 Mycobacterial growth conditions

M. tuberculosis H37Rv mutants were grown on Middlebrook 7H10-10%OADC containing 25 µg/ml of kanamycin at 37 °C for 3-4 weeks. *M. smegmatis* mc^2155 was grown on LB agar or in LB broth supplemented with 0.05% (w/v) Tween 80 at 37 °C with the shaking speed of 200 rpm for 2-3 days. Transformants of *M. smegmatis* mc^2155 containing candidate genes were grown on LB agar containing 50 µg/ml of kanamycin.

3.5.1.2 *E. coli* growth conditions

E. coli was grown on LB (Appendix B) agar at 37 °C for overnight or in LB broth at 37 °C with shaking speed of 250 rpm for overnight. Transformants of *E. coli* were grown on LB agar containing 50 µg/ml of kanamycin, 50 µM of IPTG and 80 µg/ml of X-gal for blue/white screening of recombinant colonies and incubated at 37 °C overnight.

3.5.2 DNA isolation

3.5.2.1 Genomic DNA isolation of *M. tuberculosis* H37Rv mutants

One-loop full of *M. tuberculosis* H37Rv colonies grown on solid medium was scraped and suspended in 500 µl of TE buffer [10mM Tris-HCl, 1 mM EDTA (pH8.0)]. Cells were washed in 500 µl of TE buffer and subsequently inactivated by heating at 80 °C for 20 min. The cell suspension was centrifuged at 6,000 xg for 5 min. Cell pellet was resuspended in 400 µl of Tris-EDTA-Tween-lysozyme solution [10 mM Tris-HCl, 1 mM EDTA (pH 8.0), 0.5% (v/v) Tween 80, 2 mg/ml lysozyme] and incubated at 37 °C for 3 hr. SDS and proteinase K were added into the cell suspension to final concentrations of 1% (w/v) and 1 mg/ml, respectively. After incubation at 37 °C for 1 hr, 80 µl of 5 M NaCl and 80 µl of 10% w/v of CTAB in 0.7 M NaCl were added and the suspension was heated at 65 °C for 15 min. Proteins were extracted by adding an equal volume of chloroform-isoamylalcohol (24:1) (v/v) and mixed vigorously. Centrifugation was performed to separate the aqueous phase at 12,000 xg for 5 min. The aqueous phase was re-extracted once with the equal volume of chloroform-isoamylalcohol (24:1) (v/v). DNA was precipitated by adding 0.1 volumes of 3 M Sodium acetate and 2.5 volumes of ice chilled absolute ethanol. After incubation at -70 °C for 30 min, DNA was pelleted by centrifugation at 12,000 xg for 15 min. The pellet was washed once with 500 µl of ice-chilled 70% ethanol, dried

and resuspended in 20 µl of TE buffer. RNA was digested by adding 1 µl of 10 mg/ml RNaseA. After incubation at 37 °C for 1 hr, 2 µl aliquot of genomic DNA was analyzed using 0.8% agarose gel electrophoresis. DNA was stored at -20 °C until use.

3.5.2.2 Crude DNA isolation

One loopful of *M. tuberculosis* H37Rv, *M. smegmatis* mc^2 155 and *E. coli* DH5α colonies was scraped from the agar plate and suspended in 200 µl of TE buffer. The cell suspension was boiled for 20 min before centrifugation at room temperature at 12,000 xg for 3 min. Supernatant was transferred to a new microcentrifuge tube and used for polymerase chain reaction.

3.5.2.3 Plasmid DNA isolation of *E. coli*

Using QIAprep® Miniprep Kit , a single colony of *E. coli* was inoculated into 5 ml of LB broth supplemented with 50 µg/ml of kanamycin, and incubated at 37 °C with shaking at 250 rpm for overnight. Cells were harvested by centrifugation at 12,000 xg for 1 min and resuspended in 250 µl of buffer P1. Two-hundreds and fifty microlitres of buffer P2 was added and gently mixed by inversion. After that, 350 µl of buffer N3 was added and the suspension was immediately mixed by inversion. Cell debris was pelleted by centrifugation at 12,000 xg for 10 min and supernatant was transferred to QIAprep spin column. The column was centrifuged at 12,000 xg for 1 min and flow through was discarded. To wash the column, 750 µl of PE buffer was added and the column was centrifuged at 12,000 xg for 1 min. The flow through was discarded and the column was recentrifuged again for 1 min. In order to elute plasmid DNA, column was placed in a new microcentrifuge tube and 50 µl of EB buffer was added to the center of the column. Centrifugation was performed at 12,000 xg for 1 min. Aliqout of plasmid DNA was analyzed using 0.8% agarose gel electrophoresis.

3.5.3 Agarose gel electrophoresis

For standard minigels, 0.8% agarose was prepared by dissolving 0.16 g of powdered agarose in 20 ml of 1xTBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA adjusted pH to 8.3) in Erlenmeyer flask. The slurry was heated in a microwave oven until the agarose completely dissolves. GelStar Stain solution (10,000X) was added to the agarose gel to make a final concentration of 1x and mixed by gentle swirling. An appropriate comb was chosen for forming the sample slots in the gel; the comb position should be 0.5-1.0 cm above the plate. The warm agarose gel was poured into the mold, allowed it to set completely (30-45 min at a room temperature). Before electrophoresis, the gel was transferred to the electrophoresis tank and 1x

TBE buffer was filled to cover the gel. Sample DNA was mixed with dye and loaded into the wells. Five hundreds ng of standard DNA fragments (λ DNA/*Hin*dIII fragments or 100 bp ladder DNA) was used as DNA size marker. The electric current of 8 V/cm was applied to the gel. After electrophoresis, the gel was illuminated under an ultraviolet light.

3.5.4 Primers

3.5.4.1 Primers for transposon localization

To study the localization of transposon in the *M. tuberculosis* H37Rv genome, a strategy of DNA Walking using the *SpeedUp*™ Premix Kit (Seegene, Korea) was performed. The interested gene adjacent to the transposon sequence was amplified by three PCR reactions. The general strategy of DNA Walking ACP™ PCR technology was shown in Fig. 3.1. Three transposon specific primers (TSP1, TSP2 and TSP3) were designed for each of PCR reaction. In addition, three DNA Walking ACP (DW-ACP) primers (DW-ACP1, DW-ACP-N and Universal) were used to capture unknown target sites with high specificity. All primers sequences were shown in Table 3.1.

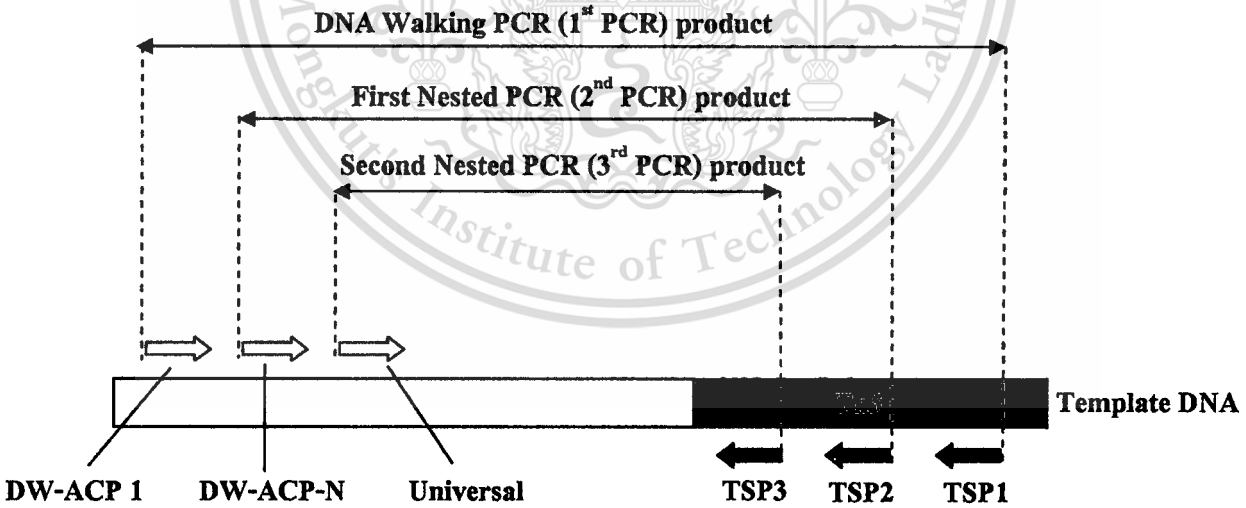


Figure 3.1 The general strategy of DNA Walking ACP™ PCR technology

Table 3.1 Primers used for transposon localization

Reaction	Primer name	Sequences (5' to 3')
First	TSP1	GCCAGTTTAGTCTGACCATC
	DW-ACP1	ACP-AGGTC
Second	TSP2	GGCGCATCGGGCTTCCCATACAAT
	DW-ACP-N	ACPN-GGTC
Third	TSP3	ATTGCCCGACATTATCGCGAGCCC
	Universal	TCACAGAAGTATGCCAAGCGA

3.5.4.2 Primers for sequencing

The recombinant plasmids pDrive carrying each of PCR products, Tn-77, Tn-196, pDrive-Rv0470A, and pDrive-*ksgA*, were sequenced using T7 promoter primer as the sequencing primer. For confirming the presence of transposon in each of candidate genes, Tn*5*-1 primer designed from Tn5 transposon position was used as another sequencing primer. For determination of the correct nucleotide sequence of the *M. tuberculosis* Rv0470A and *ksgA* gene, which was cloned into the replicative plasmid pMV261 to generate pMV261-Rv0470A and pMV261-*ksgA*, the pMV261-FBam and pMV261-FXba primers were used as sequencing primers for sense- and antisense-strands, respectively. Nucleotide sequences of primers were shown in Table 3.2.

Table 3.2 Primers used for sequencing in this study

Primer name	Sequences (5' to 3')
T7 promoter primer	TAATACGACTCACTATAGGG
Tn*5*-1 primer	TCAGAAACAACTCTGGCGCATCGGG
pMV261-FBam primer	AGGAATCACTTCGCAAT
pMV261-FXba primer	TACGTGGCGAACTCCGTTGT

3.5.4.3 Primers for amplification of Rv0470A and *ksgA* genes of *M. tuberculosis* H37Rv

Nucleotide sequences of Rv0470A and *ksgA* of *M. tuberculosis* H37Rv were derived from the Genbank database (http://www.ncbi.nlm.nih.gov/sites/genome) (Accession No. AL123456). Primers were designed to contain the restriction enzyme recognition sites at 5' ends to facilitate a directional cloning. The location and sequences of the forward and reverse primers

were shown in Fig. 3.2. The *Bam*HI (GGATCC) recognition site was located at 5' end of forward primers, *Bam*HI-Rv0470A F and *Bam*HI-*ksgA* F primers, and *Hin*dIII (AAGCTT) was used for reverse primers, *Hin*dIII-Rv0470A R and *Hin*dIII-*ksgA* R primers.

Start

GGATCCGTGG GCGCGGGCGG TTGGGAGGTC GTGCTGGCGT CGCTCCCCTA
CGGGCTGTTG TGCACCACGG TGCTGATGGG TAAGCACATC GACAAGATCG
GTTACGACGA .....GCTGTGGTAT
GCCGCGTTGG CCTGGCTGCA CGTGCGTCAG GCCGGTGCGC TGCTGGTTGT
GGGCCTGGCG ATCGGTGCCT GGCGGCGCGC CTGGTGATTT TCGCTGAAAG CTT

Stop

*Bam*HI-Rv0470A F : 5'-GGATCCGTGGGCGCGGGCGGTTGG-3'

*Hin*dIII-Rv0470A R : 5'-AAGCTTTCAGCGAAAATCACCAGGCG-3'

(A)

Start

GGATCCATGT GCTGCACGAG CGGGTGCGCG CTGACCATCC GGCTGCTCGG
GCGCACTGAG ATCAGGCGGC TGGCCAAAGA GCTCGACTTT CGGCCGCGCA
AATCTCTCGG ..... ACTTCGTGCG
GCTGCTGCGA CGGTCCGGCG GCTCCGACGA GGCCACCAGC ACCGGCCGGG
ACGCCAGGGC GCCGGACATT TCGGGCACG CGTCGGCGAG CTGAAAGCTT

Stop

*Bam*HI-*ksgA* F : 5'-GGATCCATGTGCTGCACGAGCG-3'

*Hin*dIII-*ksg*A R : 5'-AAGCTTTCAGCTCGCCGACGCGT-3'

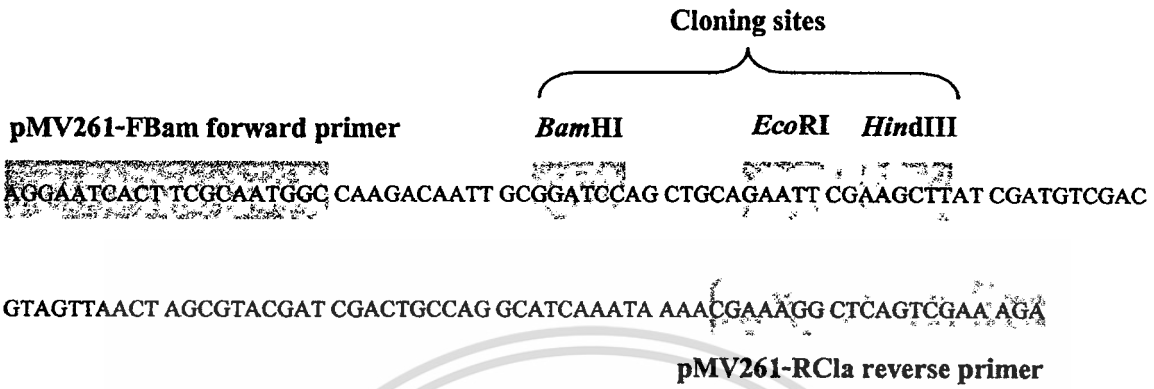
(B)

Figure 3.2 Partial sequences of *M. tuberculosis* Rv0470A (A) and *ksgA* (B) gene and location of primers *Bam*HI-Rv0470A F, *Hin*dIII-Rv0470A R, *Bam*HI-*ksgA* F, and *Hin*dIII-*ksg*A R

3.5.4.4 Primers for determination of inserted DNA fragment in recombinant plasmid pMV261

pMV261-FBam (5'-AGGAATCACTTCGCAAT-3') and pMV261-RCla (5'-TCTTTCGACTGAGCCTTTCG-3') primers were derived from a nucleotide sequence of pMV261 (download from http://www.einstein.yu.edu/tbresearch/Resources/Vectors/pMV261/261.gbk.txt) and located upstream and downstream of the multicloning sites (Fig. 3.3) These

primers were used in PCR reaction to determine the presence of inserted DNA in the recombinant plasmid pMV261.



pMV261-FBam forward primer : 5'-AGGAATCACT TCGCAATGGC-3'

pMV261-RCla reverse primer : 5'-TCTTTCGACTGAGCCTTTCG-3'

Figure 3.3 Partial nucleotide sequence of the multicloning site of pMV261. Location of pMV261-FBam and pMV261-RCla primers and the recognition sequence of *Bam*HI, *Eco*RI, and *Hin*dIII were as indicated in grey boxes

3.5.5 DNA amplification by PCR

3.5.5.1 Amplification of unknown genes flanking to the transposon region

Unknown genes flanking to the transposon region was PCR amplified by using DNA Walking *SpeedUp*™ Premix Kit. In the first PCR reaction, 50 µl reaction mixture contained 1X SeeAmp™ ACP™ Master Mix II, and 0.2 µM each of TSP1 and DW-ACP1 primers. DNA amplification was performed with the following condition: 1 cycle of 94°C for 5 min, 42 °C for 1 min, and 72 °C for 1 min; 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 100 sec; 1 cycle of 72 °C for 7 min.

PCR product from the first PCR reaction was purified using QIAquick® PCR Purification Kit (Section.3.5.6.1) and used as template in the second PCR. For second and third PCR reaction, 20 µl reaction mixture comprised of 1X SeeAmp™ ACP™ Master Mix II, and 0.5 µM each of TSP2 and DW-ACP-N primers (for second PCR) and TSP3 and Universal primers

(for third PCR) respectively. PCR was performed as follows: 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 100 sec; 1 cycle of 72 °C for 7 min. PCR product was purified and analyzed using 0.8% agarose gel electrophoresis.

3.5.5.2 Amplification of Rv0470A and *ksgA* genes of *M. tuberculosis* H37Rv

Rv0470A and *ksgA* genes of *M. tuberculosis* H37Rv was amplified with *Bam*HI-Rv0470A F/*Hin*dIII-Rv0470A R primers and *Bam*HI-*ksgA* F / *Hin*dIII-*ksg*A R primers respectively using crude DNA of *M. tuberculosis* H37Rv as template. Fifty µl of PCR reaction contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 0.5 µM each of primers, 0.05U of *Taq* polymerase (Promega, Madison, USA), and 50 ng of crude DNA. The reaction parameters consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The final cycle was followed by an additional extension at 72 °C for 7 min. PCR product was analyzed using 0.8% agarose gel electrophoresis.

3.5.5.3 PCR for confirming the presence of transposon DNA fragment

Genomic DNA of *M. tuberculosis* H37Rv mutants was amplified by using *Bam*HI-Rv0470A F / Tn*5*-1 primers for Tn-77 and *Bam*HI-*rpfB* F (5'-GGATCCATGTTGCGCCTGGTA GTC-3') / Tn*5*-1 primers for Tn-196 respectively. The PCR component and amplification profile were applied similar to that as described in the section 3.5.5.2.

3.5.5.4 PCR for determining inserted DNA fragment in recombinant plasmid pMV261

The recombinant plasmid pMV261 containing Rv0470A or *ksgA* was detected by PCR using pMV261-FBam and pMV261-RCla primers (Fig. 3.3). Crude DNA from each transformant was prepared (Section 3.5.2.2) and used as template in PCR reaction. PCR reaction was achieved using the following parameters: 1 cycle of 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The final cycle was followed by an additional extension at 72 °C for 7 min. PCR product was analyzed using 0.8% agarose gel electrophoresis.

3.5.6 DNA purification

3.5.6.1 Purification of PCR product

PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Germany). Briefly, 5 volumes of PBI buffer were added to PCR product and the mixture was mixed. A QIAquick spin column was placed in a provided 2 ml collection tube; sample was applied to the QIAquick column and the column was centrifuged at 12,000 xg for 30-60 sec. Flow-through was

discarded. The column was then placed back into the collection tube. Centrifugation was repeated after adding 750 µl of PE Buffer to wash the column. The flow-through was discarded and the column was placed back into the collection tube. The column was centrifuged for an additional 1 min and placed into a clean 1.5 ml-microcentrifuge tube. DNA was eluted from the column by adding 30-50 µl of EB buffer to the center of the membrane and column was centrifuged for 1 min. Aliquot of purified PCR product was analyzed using 0.8% agarose gel electrophoresis.

3.5.6.2 Purification of DNA fragment from agarose gel

DNA fragment was purified using the QIAquick Gel Extraction Kit (Qiagen, Germany). In brief, DNA fragment was excised from agarose gel and transferred to a microcentrifuge tube. Three volumes of gel weight of QG buffer were added and gel was incubated at 50 °C for 10 min or until gel was completely dissolved. Afterthat, the gel solution was transferred into QIAquick column and the column was centrifuged at 12,000 xg for 1 min. Flow-through was discarded. To wash the column, 750 µl of PE buffer were added and centrifugation was performed at 12,000 xg for 1 min. The flow through was discarded and the column was re-centrifuged for an additional 1 min at 12,000 xg. QIAquick column was placed into a microcentrifuge tube. DNA was eluted by adding 30 µl of EB buffer to the center of the QIAquick membrane and the column was centrifuged for 1 min. The purified DNA was analyzed by gel electrophoresis.

3.5.7 Ligation reaction

3.5.7.1 Ligation of PCR product to TA-cloning vector

Purified PCR product was ligated to plasmid pDrive (Qiagen, Germany) by setting up a ligation reaction as shown in Table 3.3. The ligation reaction was incubated at 16 °C overnight and transformed into competent cell *E. coli* strain DH5α.

Table 3.3 Ligation reaction of PCR product and plasmid pDrive

Components	Volume (µl)
Plasmid pDrive (50 ng/µl)	1
Purified PCR product (40 ng/µl)	4
2X ligation buffer containing T4 ligase(0.15 U/µl)	5
Total	10

3.5.7.2 Ligation of amplified Rv0470A and *ksgA* to pMV261

After digestion of amplified Rv0470A, *ksgA*, and pMV261 with *Bam*HI and *Hin*dIII (Section 3.5.10.2) and purification with QIAquick Gel Extraction Kit (Section 3.5.6.2), the ligation reaction was performed as shown in Table 3.4. After incubation at 20 °C for 4 hr, the ligation reaction was transformed into competent cells *E. coli* DH5α.

Table 3.4 Ligation reaction of pMV261 with either *M. tuberculosis* H37Rv Rv0470A or *ksgA* genes

Components	Volume (µl)
Purified *Bam*HI-*Hin*dIII digested Rv0470A or *ksgA* (50 ng/µl)	6
Purified *Bam*HI-*Hin*dIII digested pMV261(50 ng/µl)	2
T4 ligase (30 units/µl)	1
10X ligation buffer	1
Total	10

3.5.8 Preparation of competent cells

3.5.8.1 Preparation of competent cells *E. coli* DH5α

A single colony of *E. coli* strain DH5α was picked up from LB agar plate and inoculated into 5 ml of LB broth. The culture was incubated at 37 °C with shaking speed of 250 rpm for overnight. Three milliliters of overnight culture was inoculated into 100 ml of SOB medium (Appendix B) in a 250 ml flask and incubated at 37 °C with shaking speed of 250 rpm for 2 to 3 hr or until OD_{600} reached of 0.3-0.4. Subsequently, cells were incubated on ice for 15 min and harvested by centrifugation at 5,000 xg for 15 min at 4 °C. Cells was resuspened in 1/3 volume of RF-1 solution (Appendix B), incubated on ice for 15 min and collected by centrifugation at 5,000 xg for 15 min at 4 °C. Cells were resuspended in 1/25 volume of RF-2 solution (Appendix B) and stored at -70 °C.

3.5.8.2 Preparation of competent cells *M. smegmatis* mc^{2}155

Competent cells of *M. smegmatis* mc^{2}155 were prepared by inoculating one colony of *M. smegmatis* mc^2 155 in 20 ml of LB medium containing 0.05% Tween 80 and incubated at 37 °C with shaking speed at 200 rpm for 3-5 days. The large-scale culture was prepared by inoculating 10 ml of pre-culture into 200 ml of LB broth and incubated at 37 °C with shaking for 17 hr or until and OD_{600} reached 0.5-1.0. Cells were incubated on ice for 1.5 hr before harvesting

by centrifugation at 5,000 x g for 10 min at 4 °C. The cells were washed three times in ice-cold 10% glycerol. Finally, cells were resuspened in 1:100 original culture volume of ice-cold 10% glycerol. The competent cell should be freshly prepared before use in electrotransformation.

3.5.9 Transformation

3.5.9.1 Transformation of *E. coli* strain DH5α

The ligation reaction as described in section 3.5.7 was mixed with 100 µl of competent *E. coli* strain DH5αand incubated on ice for 30 min. The suspension was heated at 42 °C for 90 seconds and subsequently incubated on ice for 2 min. Nine hundreds microlittres of LB broth was added and cell suspension was incubated at 37 °C for 1 hr. Aliquot of 100 µl of cells was spreaded on LB agar containing 50 µg/ml of kanamycin, 50 µM of IPTG and 80 µg/ml of X-gal for blue/white screening of recombinant clones. Plates were incubated at 37 °C overnight.

3.5.9.2 Transformation of *M. smegmatis* mc^{2}155

Approximate 1 µg of recombinant plasmid DNA was mixed with 100 µl of competent cells. The cell suspension was kept on ice for 5 min and transferred to a 0.2 cm electrode-gap electroporation cuvette. The cuvette was placed on electroporation chamber and subjected to one single pulse of 2.5 kV, 25 µF with the pulse-controller resistance setting at 1,000 ohms. After electroporation, a cuvette was stood on ice for 10 min and added with 1 ml of LB broth. The suspension was then transferred to a sterile 15 ml tube and incubated at 37 °C for 2 hr. A total of 200 µl of cell suspension was spreaded on LB plate containing 50 µg/ml of kanamycin and plates were incubated at 37 °C for 3-5 days.

3.5.10 Restriction enzyme digestion

3.5.10.1 Digestion of recombinant plasmid pDrive

The recombinant plasmid pDrive was examined by digesting with *Eco*RI. The digestion reaction was set as described in Table 3.5 and incubated at 37 °C for overnight. The digested product was then analyzed using 1.0% agarose gel electrophoresis. The recombinant plasmid pDrive containing expected size of DNA fragment was subsequently submitted for DNA sequencing.

Table 3.5 Digestion reaction of recombinant plasmid pDrive

Components	Volume (µl)
Recombinant plasmid DNA (60 ng/µl)	3
*Eco*RI (12 units/µl)	1
10X *Eco*RI buffer	1
BSA (0.1 mg/ml)	1
Deionized water	4
Total	10

3.5.10.2 Digestion of recombinant plasmids pDrive-Rv0470A, pDrive-*ksgA*, and pMV261

Recombinant plasmids pDrive-Rv0470A, pDrive-*ksgA* and mycobacterium shuttle vector pMV261 were prepared using the QIAprep Spin Miniprep Kit as described in Section 3.5.2.3. They were digested with *Bam*HI and *Hin*dIII as described in Table 3.6. The reaction was incubated at 37 °C for overnight and the digested product was subsequently analyzed using 1% agarose gel electrophoresis. After that, DNA fragments of Rv0470A, *ksgA* and pMV261 were gel-purified and analyzed using the QIAquick gel extraction Kit (Section 3.5.6.2) and 1% agarose gel electrophoresis respectively. The purified DNA fragments were then used in the ligation reaction as described in section 3.5.7.2.

Table 3.6 Digestion reaction of pDrive-Rv0470A, pDrive-*ksgA* and pMV261

Components	Volume (µl)		
	pDrive-Rv0470A	pDrive-*ksgA*	pMV261
pDrive-Rv0470A (50 ng/µl)	50	-	-
pDrive-*ksgA* (50 ng/µl)	-	50	-
pMV261 (50 ng/µl)	-	-	30
*Bam*HI (12 units/µl)	4	4	5
*Hin*dIII (12 units/µl)	4	4	5
10X buffer	8	8	5
BSA (0.1 mg/ml)	8	8	5
Deionized water	6	6	-
Total	80	80	50

3.5.10.3 Digestion of recombinant plasmid pMV261

The recombinant plasmid pMV261 carrying either Rv0470A or *ksgA* was confirmed for its presence of inserted DNA by digesting with *Cla*I, *Kpn*I and *Xba*I. The digestion reaction was set as described in Table 3.7 and incubated at 37 °C for overnight, before analyzing with 1% agarose gel electrophoresis. The recombinant plasmid containing the expected insert DNA fragment was subsequently submitted for DNA sequencing.

Table 3.7 Digestion reaction of pMV261-Rv0470A and pMV261-*ksgA* with restriction enzymes

Components	Volume (µl)	
	pMV261-Rv0470A	pMV261-*ksgA*
pMV261-Rv0470A (50 ng/µl)	3	-
pMV261-*ksgA* (50 ng/µl)	-	3
*Cla*I (12 units/µl)	1	-
*Kpn*I (12 units/µl)	-	1
*Xba*I (12 units/µl)	1	-
10X buffer	1	1
BSA (0.1 mg/ml)	1	1
Deionized water	3	4
Total	10	10

3.5.11 Nucleotide sequencing

Recombinant plasmid DNA and PCR products were sequenced with the Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) and ABI PRISM® 3700 DNA analyzer at First BASE Laboratories (Malaysia). All primers for sequencing were shown in Table 3.2. Nucleotide sequences of recombinant plasmid DNA and PCR products were analyzed by comparing with *M. tuberculosis* H37Rv sequence deposited in Genbank database (Accession No. AL123456).

3.5.12 Drug susceptibility test

3.5.12.1 Clarithromycin susceptibility testing of *M. smegmatis* on solid medium

Colonies of *M. smegmatis* transformants grown on kanamycin containing LB agar were scraped and resuspended in 500 µl of LB broth. Five microlittres of the cell suspension was streaked on LB agar containing different concentrations of clarithromycin (0, 5, 10, 15 and 20 µg/ml) and 50 µg/ml of kanamycin. The plates were incubated at 37 °C for 3-5 days. Growth of each tested strain was compared with that of the control strain *M. smegmatis*::pMV261.

3.5.12.2 Determination of Minimum Inhibition Concentration (MIC) using microdilution method

Cell suspension of *M. smegmatis*, *M. smegmatis*::pMV261, *M. smegmatis*:: pMV261-Rv0470A and *M. smegmatis*::pMV261-*ksgA* was prepared in Middlebrook 7H9 supplemented with 10%OADC to the turbidity of McFarland No.1 (about 3 x 10^7 cells/ml). The cell suspension were 10-fold diluted to be 3 x 10^5 cells/ml and used 100 µl per well of microtitreplate. For each well, a total of 200 µl suspension contained 1.5 x 10^5 cells/ml of inoculum and each concentration of clarithromycin ranged from 0 to 512 µg/ml (0, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml). The plate was covered with a lid and sealed with parafilm. Incubation was carried out at 37 °C in a moisture box. Result was determined visually and the plate should be checked everyday for growth and contamination until 5-7 days of incubation. MIC was determined by the lowest concentration that inhibits the visible growth when growth in control well (0 µg/ml of clarithromycin) was appeared (Woods *et al.*, 2003).

CHAPTER 4

RESULTS

4.1 Localization of transposon on genome of macrolide-susceptible *M. tuberculosis* H37Rv mutants Tn-77 and Tn-196

M. tuberculosis H37Rv mutants Tn-77 and Tn-196 were subcultured and genomic DNA isolation was done for amplification of the DNA flanking to the inserted transposon Tn*5* using genome walking technique. PCR products were purified, ligated with TA cloning vector and transformed into *E. coli* DH5α. Transformants were selected for plasmid DNA isolation, digestion by restriction endonucleases and DNA sequencing. For mapping of transposon DNA fragment, nucleotide sequences of DNA flanking to transposon were compared with nucleotide sequence of *M. tuberculosis* H37Rv.

4.1.1 Genomic DNA isolation of *M. tuberculosis* H37Rv mutants Tn-77 and Tn-196

M. tuberculosis H37Rv mutants Tn-77 and Tn-196 were grown on Middlebrook 7H10 agar as described in 3.5.1.1. Cells were harvested and genomic DNA was isolated as described in section 3.5.2.1. Genomic DNA of Tn-77 and Tn-196 DNA were analyzed using 0.8% agarose gel electrophoresis as described in section 3.5.3. It was shown that only one dense DNA band was found in both genomic DNA mutants (Fig 4.1). These DNA sizes were shown higher than 23,130 bp of standard DNA marker λ/*Hin*dIII (Fig 4.1).

4.1.2 Amplification of DNA flanking to the transposon insertion site

Genomic DNA of *M. tuberculosis* H37Rv mutants Tn-77 and Tn-196 were used as template DNA in PCR reaction using DNA Walking *SpeedUp*™ Premix Kit as described in section 3.5.5.1 and primers shown in Table 3.1. After amplification, the 3rd PCR product was analyzed using 1% agarose gel electrophoresis. As shown in Fig. 4.2, PCR products of Tn-77 and Tn-196 were 700 bp (lane 1) and 900 bp (lane 2) respectively.

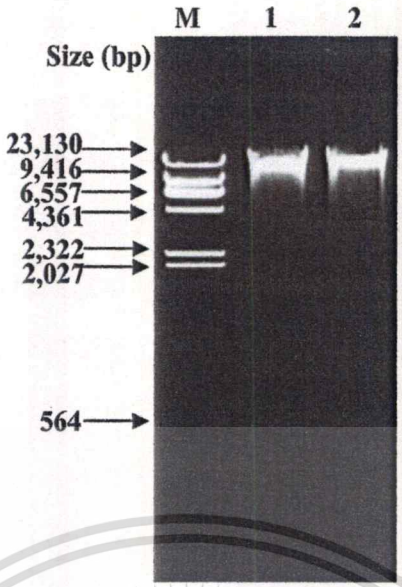


Figure 4.1 Analysis of genomic DNA of *M. tuberculosis* H37Rv mutants Tn-77 (lane 1) and Tn-196 (lane 2) using 0.8% agarose gel electrophoresis compared with λ DNA/*Hin*dIII fragments (lane M)

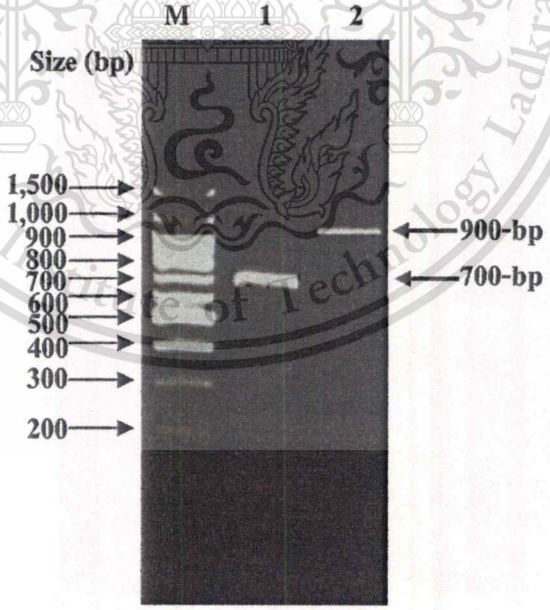


Figure 4.2 Agarose gel analysis of PCR products of Tn-77 (lane 1) and Tn-196 (lane 2) after amplification using genome walking technique compared with 100 bp DNA ladder (lane M)

4.1.3 Cloning of PCR products into the TA cloning vector

Ligation reaction and condition were shown in Table 3.3 and section 3.5.7.1. Recombinant plasmids were transformed into *E. coli* DH5α with transformation efficiency of 10^5 CFU/μg and transformants were selected using a blue/white screening method as described in section 3.5.9.1. Approximate 50 colonies were found on LB agar containing 50 μg/ml of kanamycin, 50 μM of IPTG and 80 μg/ml of X-gal. About 20% of total colonies were white in color, suggesting the presence of PCR product in these clones. Five white colonies from each transformation reaction were selected and subcultured. Plasmid DNA isolation was done for each transformant and analyzed using 0.8% agarose gel electrophoresis (Fig. 4.3 and Fig. 4.4). One transformant each (clone 1.3 and clone 1.2) showing the highest band were selected and designated as pDrive-Tn-77 and pDrive-Tn-196 respectively.

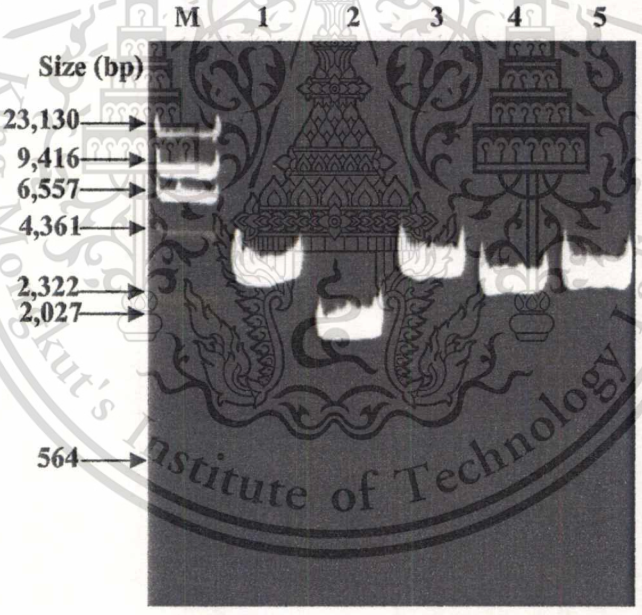


Figure 4.3 Analysis of the recombinant plasmid DNA pDrive-Tn-77 clones 1.1-1.5 (lanes 1-5) using 0.8% agarose gel electrophoresis (M = λ DNA/*Hin*dIII fragments)

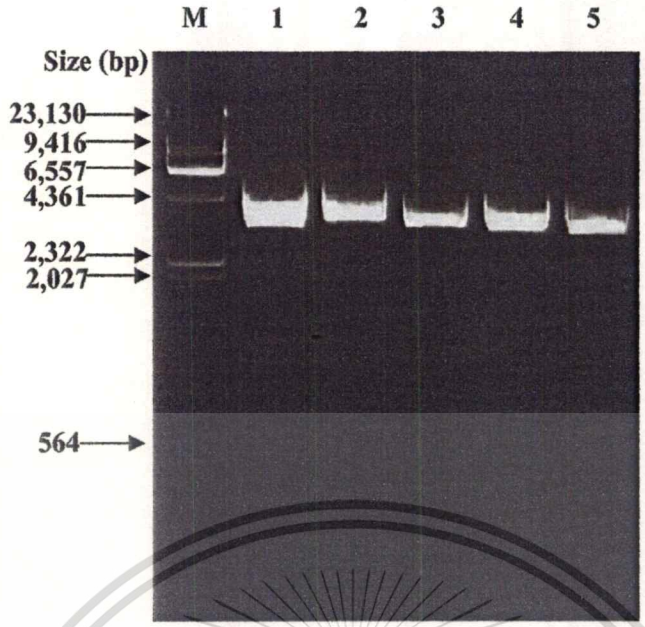


Figure 4.4 Analysis of the recombinant plasmid DNA pDrive-Tn-196 clones 1.1-1.5 (lanes 1-5) by 0.8 % agarose gel electrophoresis (M = λ DNA/*Hin*dIII fragments)

The recombinant plasmid DNA pDrive-Tn-77 and pDrive-Tn-196 were digested by *Eco*RI whose recognition site is located flanking to the insertion site of PCR product in pDrive vector (Appendix A). The digestion reaction and conditions were shown in Table 3.5 (section 3.5.10.1). After analysis using 0.8% agarose gel electrophoresis, 3,850-bp and 700-bp digested products and 3,850-bp and 960-bp digested products were obtained from pDrive-Tn-77 and pDrive-Tn-196, respectively (Fig. 4.5). Size of the digested bands is related to that of pDrive vector and PCR product respectively, suggesting the correct recombinant plasmid of pDrive-Tn-77 and pDrive-Tn-196. Subsequently, these recombinant plasmids were purified and submitted for DNA sequencing.

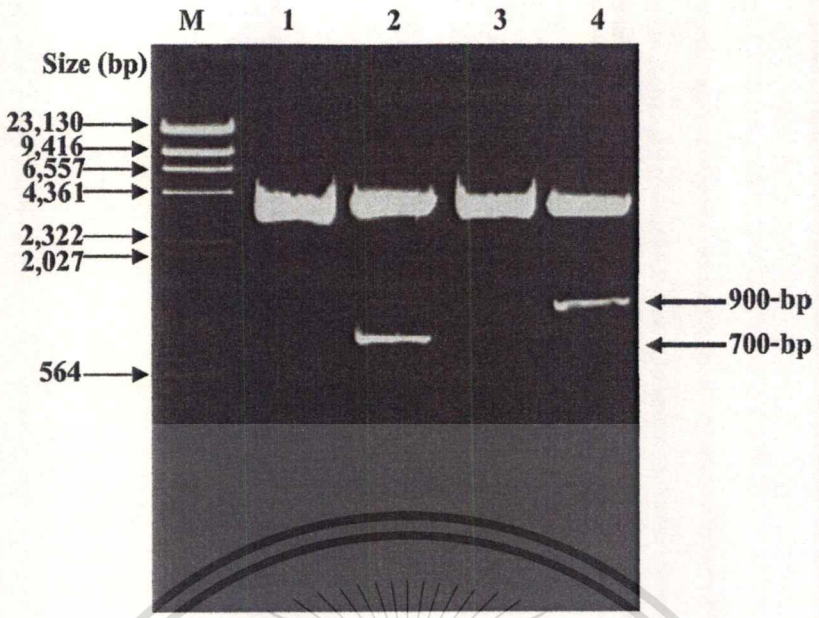


Figure 4.5 Analysis of recombinant plasmid DNA digestion by *Eco*RI using 0.8% agarose gel electrophoresis

Lane M : λ DNA/*Hin*dIII fragments

Lane 1 : Undigested pDrive-Tn-77

Lane 2 : *Eco*RI digested pDriveTn-77

Lane 3 : Undigested pDrive-Tn-196

Lane 4 : *Eco*RI digested pDriveTn-196

4.1.4 Sequencing and determination of transposon insertion site

The recombinant plasmid pDrive-Tn-77 and pDrive-Tn-196 were submitted for DNA sequencing as described in section 3.5.11 by using primer T7 promoter primer (Table 3.2) as a sequencing primer. Comparison of nucleotide sequence of DNA adjacent to the transposon DNA fragment with the *M. tuberculosis* H37Rv genome revealed that PCR product of Tn-77 showed 100% identity to a part of Rv0470A, whereas that of Tn-196 showed 100% to the DNA region between of *rpfB* and *ksgA* (Rv1009-Rv1010) (Table 4.1 and Table 4.2). Sequencing mapping of the insertion site indicated that the transposon DNA fragment was inserted in Rv0470A at nucleotide position 389-390 for Tn-77 and at *rpfB* nucleotide position 1,072-1,073 or at *ksgA* nucleotide position 11-12 for Tn-196 (Fig. 4.6).

Table 4.1 Nucleotide sequence alignment of transposon DNA fragment flanking region of pDrive-Tn-77 compared with *M. tuberculosis* H37Rv nucleotide GenBank database (Accession no. AL123456)

Protein	Gene	Identities score	Function
Hypothetical protein	Rv0470A	100%	Unknown
Member of *M. tuberculosis* PE family, Gly-, Ala-rich PGRS subfamily.	Rv1087	68%	Unknown
Plus agglutinin	(SbPRP2)	65%	Cellular processes: Toxin production and resistance
Probable PPE protein	Rv3533c	65%	Protein synthesis: Ribosomal proteins: synthesis and modification Protein fate: Degradation of proteins, peptides, and glycopeptides DNA metabolism: DNA replication, recombination, and repair Energy metabolism: Sugars Energy metabolism: Electron transport
mbtF, Mycobactin-Exochelin Synthesis (lysine ligation).	Rv2379c	65%	Protein synthesis: tRNA aminoacylation
Hypothetical protein	Rv0695	65%	Unknown
M. tuberculosis Gly-rich protein family	PE_PGRS	64%	Unknown
1,4-dihydroxy-2-naphthoate octaprenyl	*menA*	63%	Biosynthesis of cofactors, prosthetic groups, and carriers: Menaquinone and ubiquinone

Table 4.2 Nucleotide sequence alignment of transposon DNA fragment flanking region of pDrive-Tn-196 compared with *M. tuberculosis* H37Rv nucleotide GenBank database (Accession no. AL123456)

Protein	Gene	Identities score	Function
Resuscitation promoting factor B	*rpfB*	100%	RpfB is thought to promote the resuscitation and growth of dormant, nongrowing cell.
Dimethyl adenosine transferase	*ksgA*	100%	KsgA is specifically dimethylates two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 16S rRNA in the 30S particle.
Resuscitation promoting factor A	*rpfA*	68%	RpfA is thought to promote the resuscitation and growth of dormant, nongrowing cell.
Resuscitation promoting factor E	*rpfE*	66%	RpfE is thought to promote the resuscitation and growth of dormant, nongrowing cell.
Resuscitation promoting factor C	*rpfC*	63%	RpfC is thought to promote the resuscitation and growth of dormant, nongrowing cell.
PE-PGRS family protein	PE_PGRS16	59%	Unknown

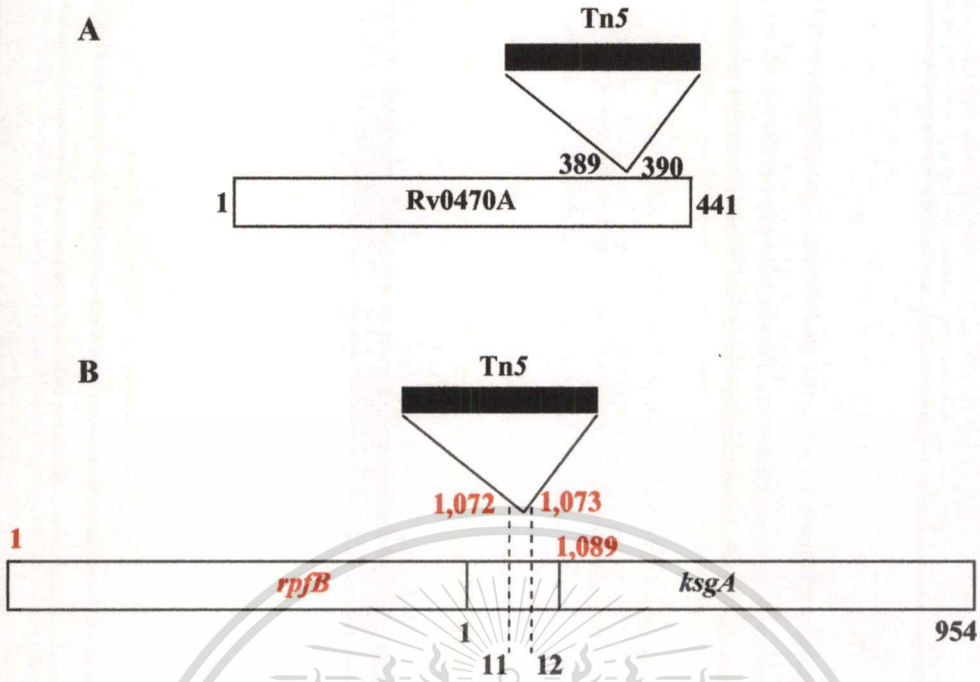


Figure 4.6 Position of transposon DNA fragment integrated in genome of macrolide-susceptible *M. tuberculosis* H37Rv mutants Tn-77 (A) and Tn-196 (B)

In order to confirm the presence of transposon DNA fragment and the insertion site, PCR was performed to amplify the transposon flanking region using genomic DNA of Tn-77 and Tn-196 mutants and primers *Bam*HI-Rv0470A F and Tn*5*-1 for amplifying Tn-77 and primers *Bam*HI-*rpfB*F and Tn*5*-1 for amplifying Tn-196 respectively. After amplification, PCR products were analyzed using 1.0% agarose gel electrophoresis. No PCR product was found in negative control that deionized water was used instead of genomic DNA as template DNA (Fig. 4.7). Results revealed approximately 700-bp and 1,400-bp PCR products for Tn-77 and Tn-196 mutants respectively (Fig. 4.7), consistent with the expected PCR products of 695 and 1,378 bp for Tn-77 and Tn-196 respectively. Subsequently, these PCR products were purified as described in section 3.5.6.2 and submitted for sequencing by using primer Tn*5*-1 as a sequencing primer. The obtained partial nucleotide sequences of Tn-77 (Fig 4.8) and Tn-196 (Fig 4.9) showed 100% identity to the Rv0470A and *rpfB-ksgA*, respectively. In addition, the results also confirmed the identical insertion site of the transposon DNA fragment in both mutants as described previously.

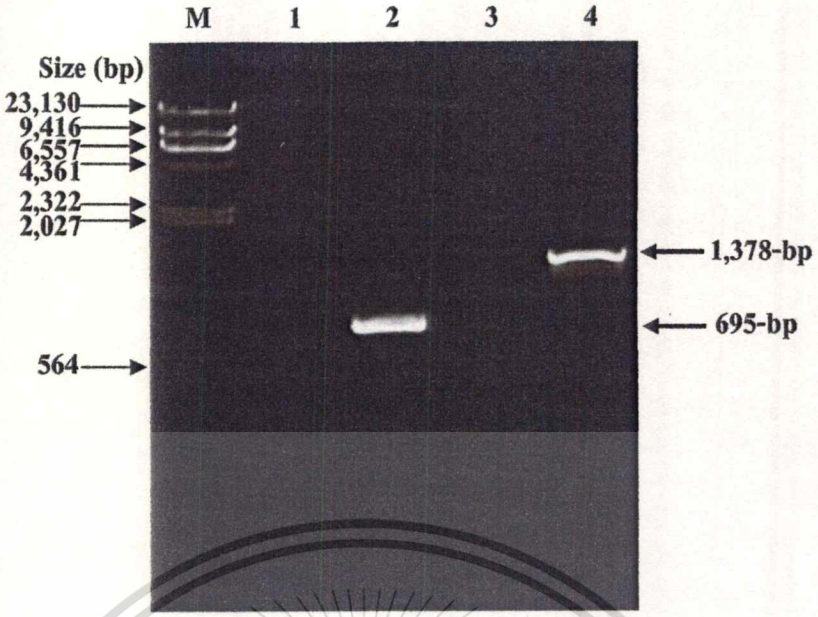


Figure 4.7 Agarose gel analysis of PCR products from genomic DNA of *M. tuberculosis* H37Rv mutants using primers *Bam*HI-Rv0470A F/Tn5-1 for Tn-77 and primers *Bam*HI-*rpfB* F/Tn5-1 for Tn-196

Lane M : λ DNA/*Hin*dIII fragments

Lane 1 : Negative control (deionized water)

Lane 2 : PCR product of Tn-77

Lane 3 : Negative control (deionized water)

Lane 4 : PCR product of Tn-196

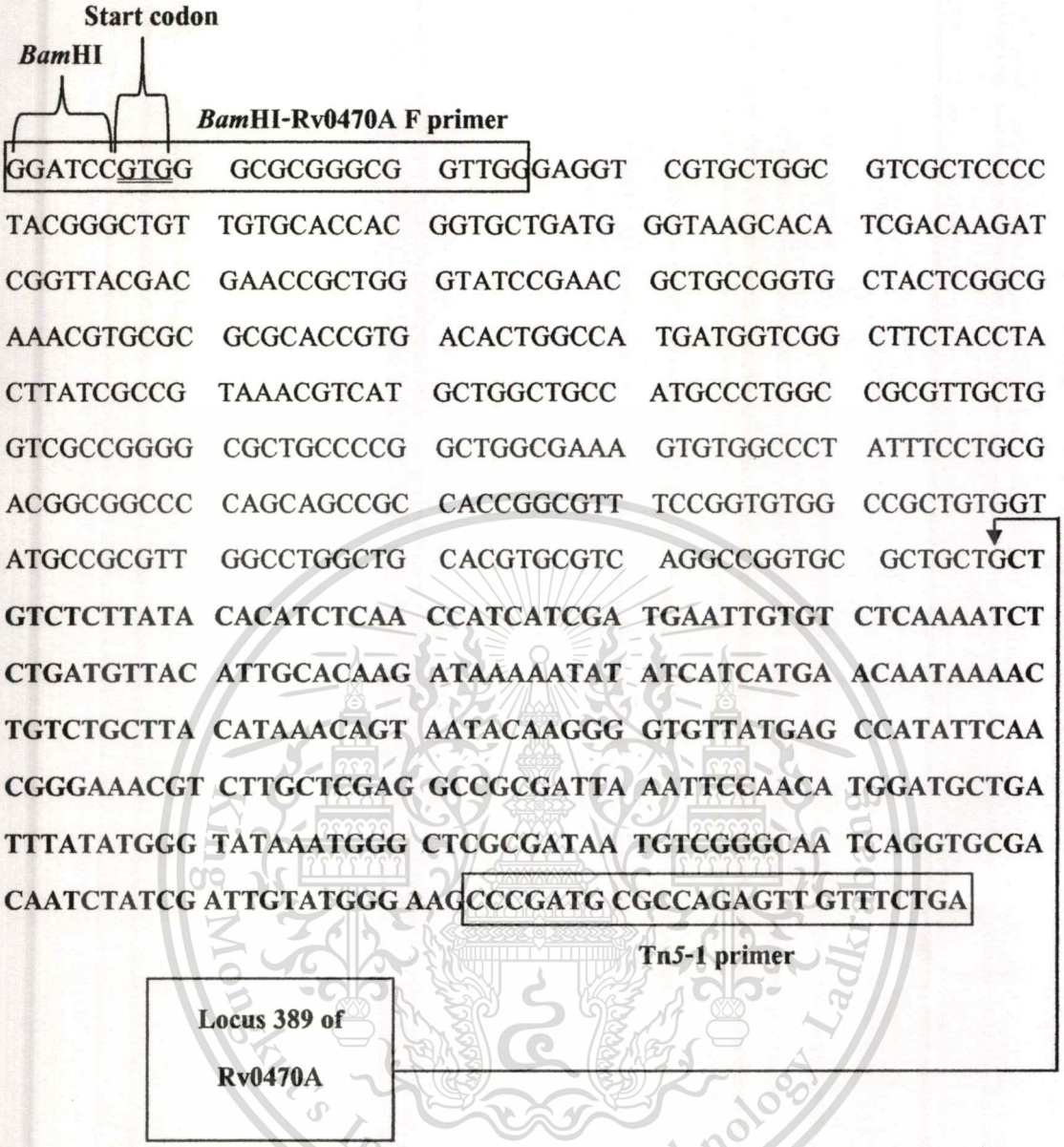


Figure 4.8 Partial nucleotide sequence of Tn-77 comprised of the Rv0470A region (389-bp), and transposon Tn*5* DNA region (bold letters) including *Bam*HI recognition site and the locations of primers are depicted in boxes.

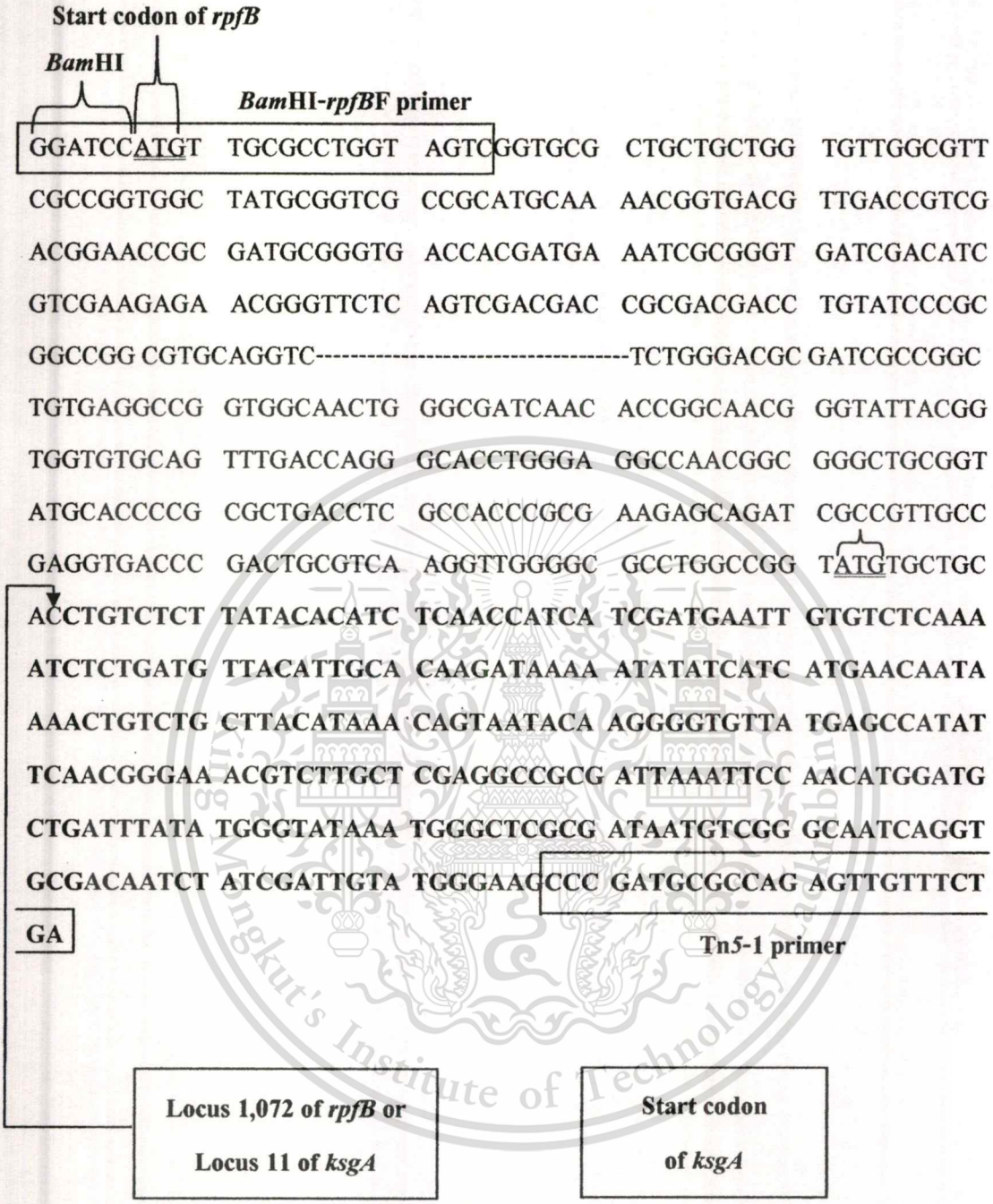


Figure 4.9 Partial nucleotide sequence of Tn-196 comprised of parts of the *rpfB* and *ksgA* (1,072-bp), transposon Tn*5* DNA region (bold letters) including *Bam*HI recognition site and the locations of primers are depicted in boxes.

4.2 Construction of the recombinant plasmid pMV261 containing Rv0470A and *ksgA* genes

Primers were designed based on the published genome sequence of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) as described in section 3.5.4.3. Rv0470A and *ksgA* were amplified from *M. tuberculosis* H37Rv crude DNA and ligated to pDrive cloning vector. The recombinant plasmid pDrive-Rv0470A and pDrive-*ksgA* were prepared for cloning into *E. coli*/*Mycobacterium* shuttle vector pMV261 by digestion with *Bam*HI and *Hin*dIII. The digested Rv0470A and *ksgA* genes fragments were ligated into the pMV261, which was previously digested by *Bam*HI and *Hin*dIII. The recombinant DNAs pMV261-Rv0470A and pMV261-*ksgA* were transformed into competent cells *E. coli* DH5α and selected on LB agar containing 50 μg/ml of kanamycin. Transformants were selected and subcultured. Plasmid DNA was isolated and analyzed by PCR, restriction enzyme digestion and sequencing.

4.2.1 PCR amplification of Rv0470A and *ksgA* of *M. tuberculosis* H37Rv

Crude DNA of *M. tuberculosis* H37Rv was prepared and used as template for PCR (Section 3.5.5.2). After amplification, the 453-bp Rv0470A and 966-bp *ksgA* products were analyzed using 1.0% agarose gel electrophoresis (Fig. 4.10). No PCR product was found in the negative control that deionized water was used instead of crude DNA as template (Fig. 4.10). Before ligation with pDrive vector, the amplified Rv0470A and *ksgA* were purified using QIAquick PCR purification Kit and determined the concentration by 1.0% agarose gel electrophoresis compared with 100 bp DNA ladder. Approximately concentration of both purified PCR products was 40 ng/ μl (Fig. 4.11).

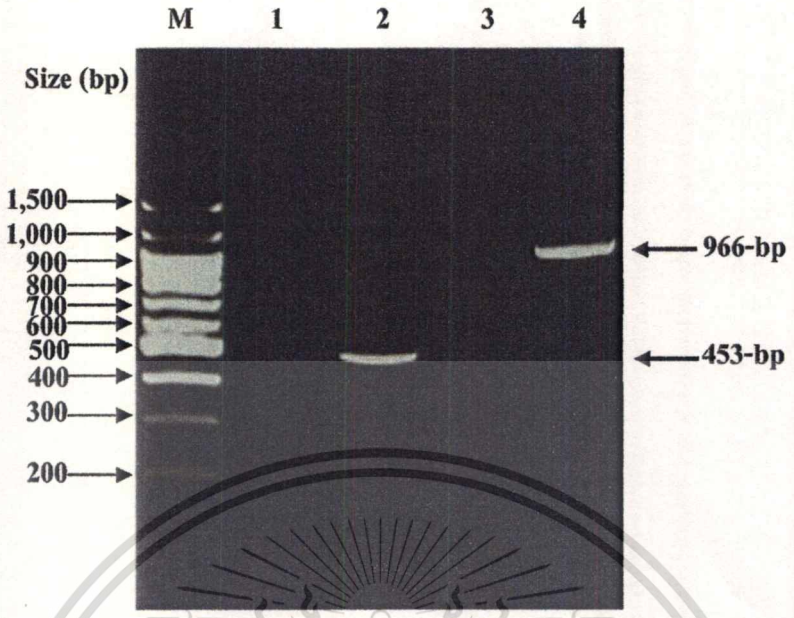


Figure 4.10 Agarose gel analysis of PCR products of *M. tuberculosis* H37Rv Rv0470A and *ksgA*

Lane M : 100 bp DNA ladder

Lane 1 : Negative control

Lane 2 : Amplified Rv0470A PCR product

Lane 3 : Negative control

Lane 4 : Amplified *ksgA* PCR product

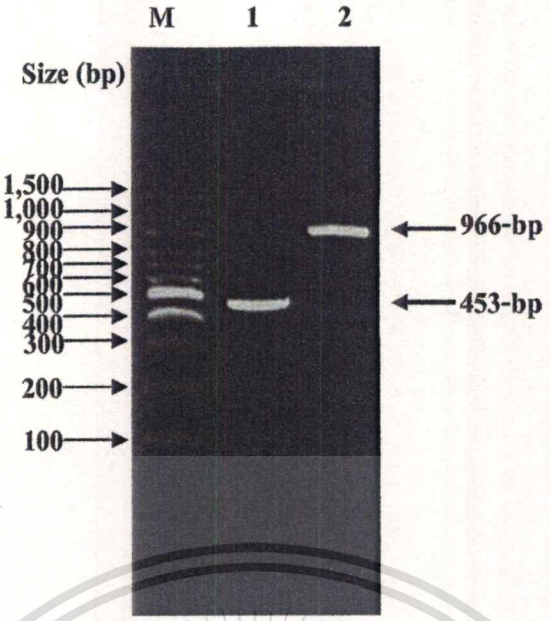


Figure 4.11 Agarose gel analysis of purified PCR products of *M. tuberculosis* H37Rv Rv0470A and *ksgA*

Lane M : 100-bp DNA ladder

Lane 1 : Purified Rv0470A

Lane 2 : Purified *ksgA*

4.2.2 Cloning of Rv0470A and *ksgA* into pDrive cloning vector

For ligation reaction, 160 ng each of purified *M. tuberculosis* H37Rv Rv0470A and *ksgA* were ligated to 50 ng of pDrive. Condition of ligation was shown in Table 3.3 (Section 3.5.7.1). The ligation product was transformed into *E. coli* DH5α and selected by using blue/ white screening. Approximate 20 colonies were found with 50% of white colonies. Four white colonies were randomly selected to subculture; plasmid DNA was isolated and analyzed using 0.8% agarose gel electrophoresis. The results were shown in Fig. 4.12 and Fig. 4.13. One each of transformant (clone 1.3 for Rv0470A and clone 1.4 for *ksgA*) was selected, plasmid extracted and *Eco*RI characterized for the expected plasmid containing a correct insertion of PCR product.

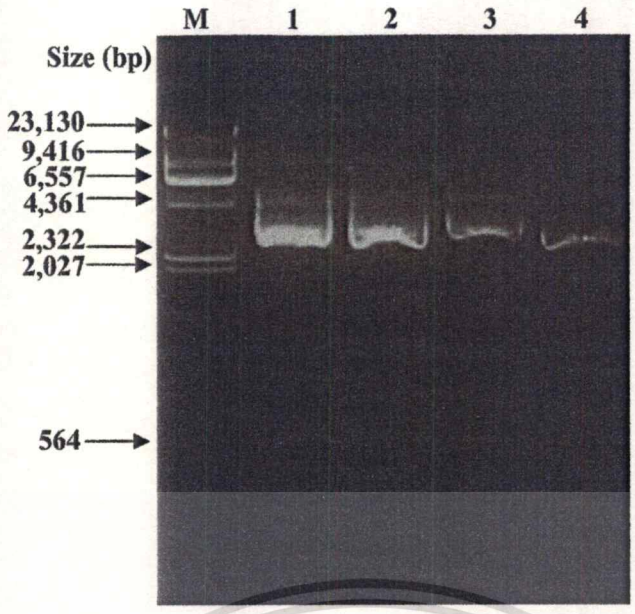


Figure 4.12 Agarose gel analysis of the recombinant plasmid DNA pDrive-Rv0470A clones 1.1-1.4 (lanes 1-4) (M = λ DNA/*Hin*dIII fragments)

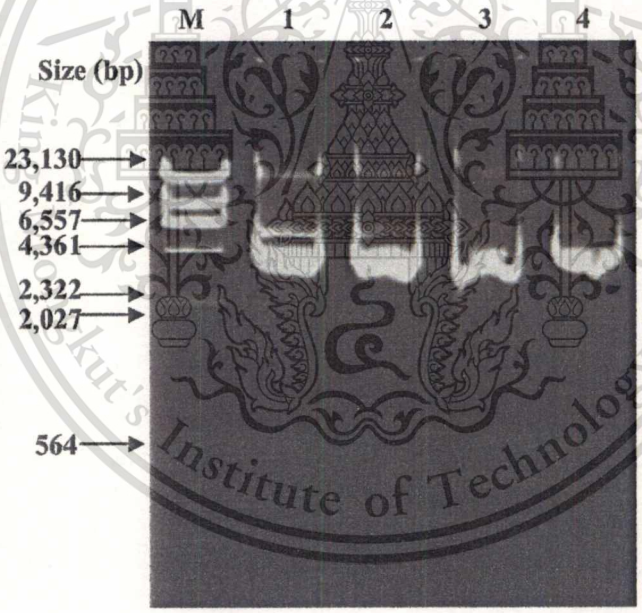


Figure 4.13 Agarose gel analysis of the recombinant plasmid DNA pDrive-*ksgA* clones 1.1-1.4 (lanes 1-4) (M = λ DNA/*Hin*dIII fragments)

Clones 1.3 and 1.4, designated as pDrive-Rv0470A and pDrive-*ksgA*, were digested with *Eco*RI as described in Table 3.5 (Section 3.5.10). After analysis using 0.8% agarose gel electrophoresis, each plasmid showed two digested products of 3,850 bp and 500 bp for pDrive-Rv0470A and of 3,850 bp and 1,000 bp for pDrive-*ksgA* (Fig. 4.14). Size of the digested DNA

bands is related to that of pDrive and each PCR product, indicating the correct recombinant plasmids as expected.

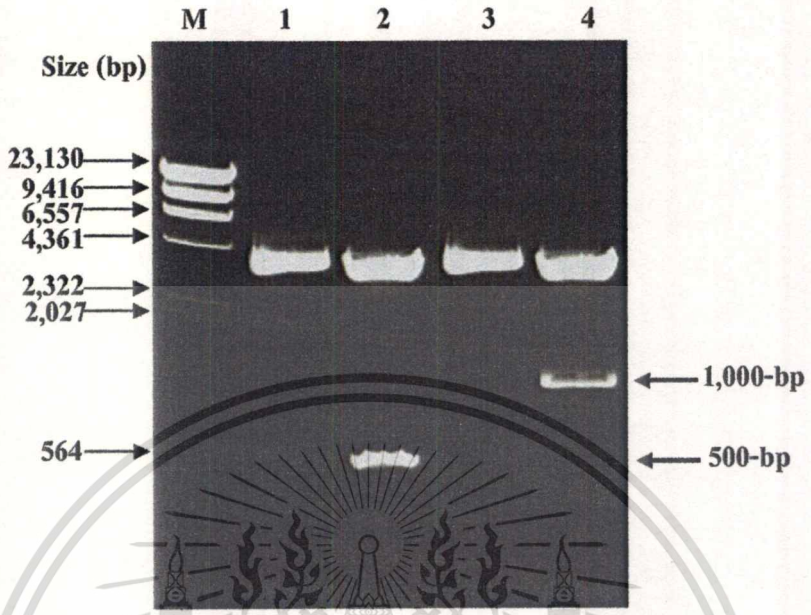


Figure 4.14 Agarose gel analysis of recombinant plasmids after digestion with *Eco*RI

Lane M : λ DNA/*Hin*dIII fragments

Lane 1 : Undigested pDrive-Rv0470A

Lane 2 : *Eco*RI-digested pDrive-Rv0470A

Lane 3 : Undigested pDrive-*ksgA*

Lane 4 : *Eco*RI-digested pDrive-*ksgA*

To confirm that each plasmid carries the amplified gene with correct nucleotide sequence, recombinant plasmids pDrive-Rv0470A and pDrive-*ksgA* were sequenced using T7 primer as a sequencing primer (Section 3.5.11). The obtained nucleotide sequence showed 100% identity to the published sequence of *M. tuberculosis* H37Rv Rv0470A and *ksgA*. In addition, the correct *Bam*HI and *Hin*dIII recognition site located in primers were identified (Fig. 4.15-4.16).

Sequence of pDrive-Rv0470A

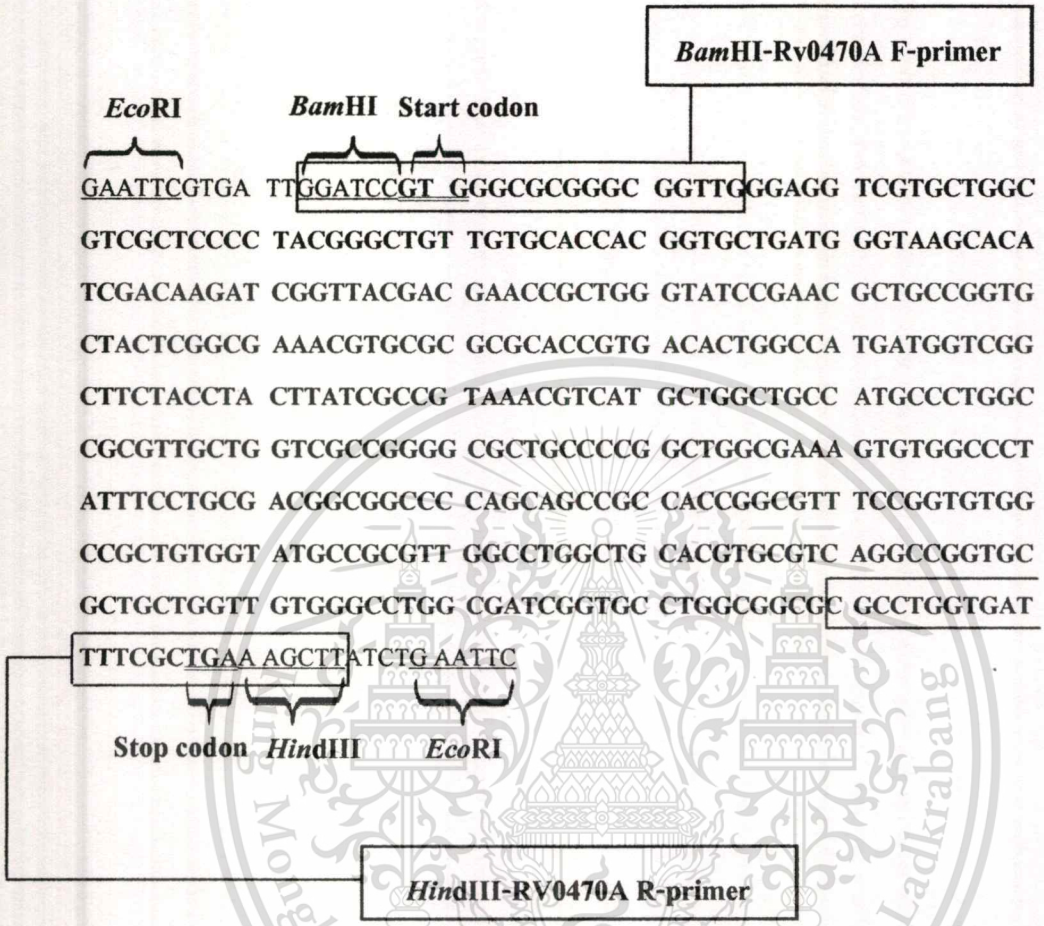


Figure 4.15 Nucleotide sequences of pDrive-Rv0470A (ORFs indicate with bold letter primers are located in boxes). A start (GTG) codon, a stop (TGA) codon and restriction enzyme recognition sites were also underlined as indicated.

Sequence of pDrive-*ksgA*

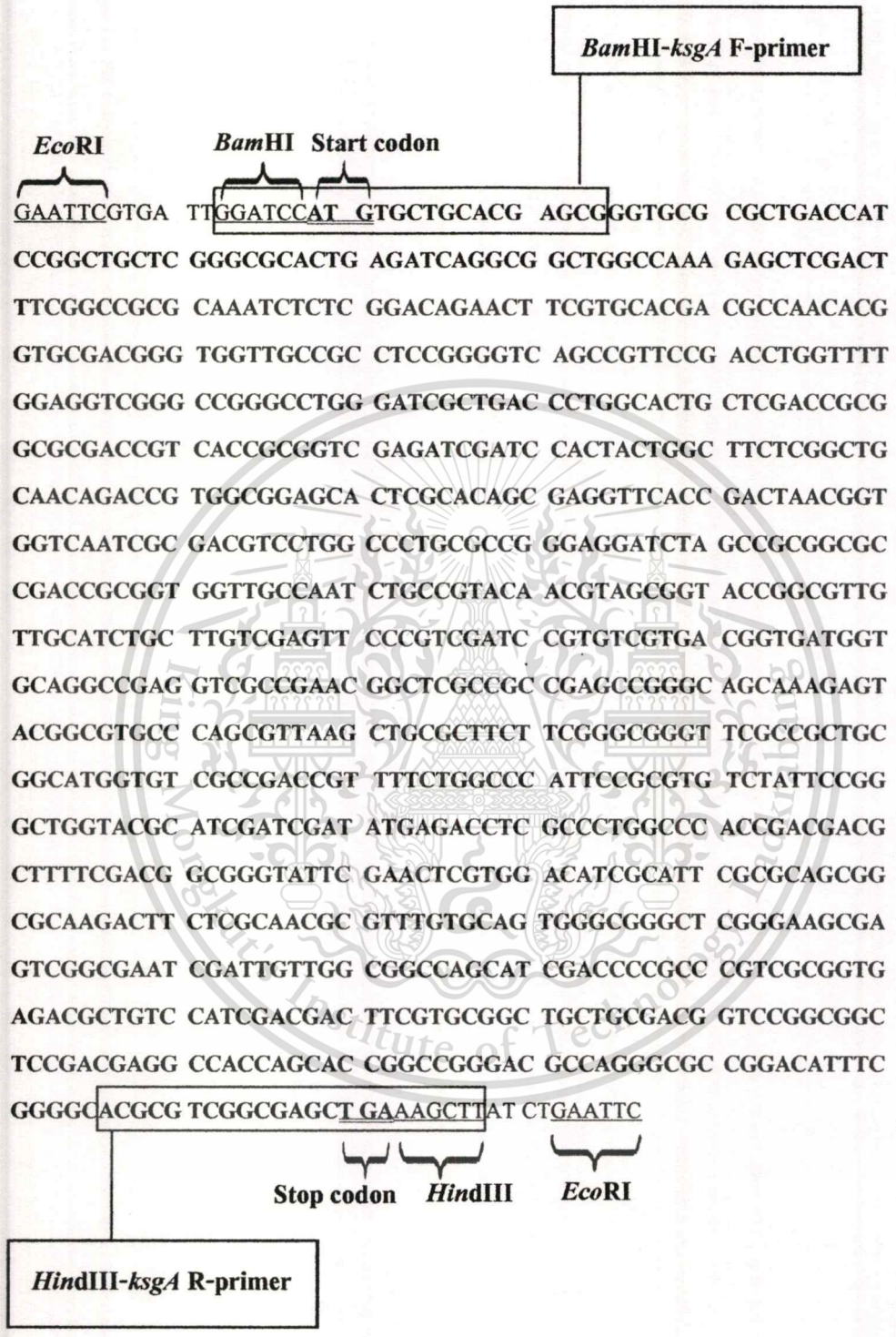


Figure 4.16 Nucleotide sequences of pDrive-*ksgA* (ORF of *ksgA* (Rv1010) is indicated in bold, whereas primers are in boxes). A start (ATG) codon, a stop (TGA) codon and restriction enzyme recognition sites were also underlined as indicated.

4.2.3 Cloning of Rv0470A and *ksgA* into plasmid pMV261

A strategy for cloning of Rv0470A and *ksgA* into pMV261 was summarized in Fig. 4.17. All plasmids were cut with *Bam*HI and *Hin*dIII; the digestion reaction and conditions were shown in Table 3.6 (Section 3.5.10.2). After digestion, a 4,462-bp linearized pMV261 was found, when analyzing with 0.8% agarose gel (Fig. 4.18), whereas *Bam*HI-*Hin*dIII digested pDrive-Rv0470A and pDrive-*ksgA* gave two digested DNA products, 3,850-bp pDrive and 453-bp Rv0470A and 3,850-bp pDrive and 966-bp *ksgA* for pDrive-Rv0470A and pDrive-*ksgA* respectively (Fig. 4.19). DNA fragments of *Bam*HI-*Hin*dIII digested pMV261, Rv0470A and *ksgA* DNA fragments were excised by using QIAquick gel extraction kit as described in section 3.5.6.2 and determined the obtained concentrations. The concentration of either purified *Bam*HI-*Hin*dIII digested plasmid pMV261, Rv0470A or that of *ksgA* were about 50 ng/µl each (data not shown). The purified DNA fragments were ligated to *Bam*HI-*Hin*dIII digested pMV261 and chemically transformed into *E. coli* DH5α with a transformation efficiency of 2.11 x 10^5 CFU/µg. Transformants were selected on LB agar containing 50 µg/ml of kanamycin. Approximate 200 colonies were found and 8 transformants were randomly selected to subculture for further analysis as described in section 3.5.1.2.

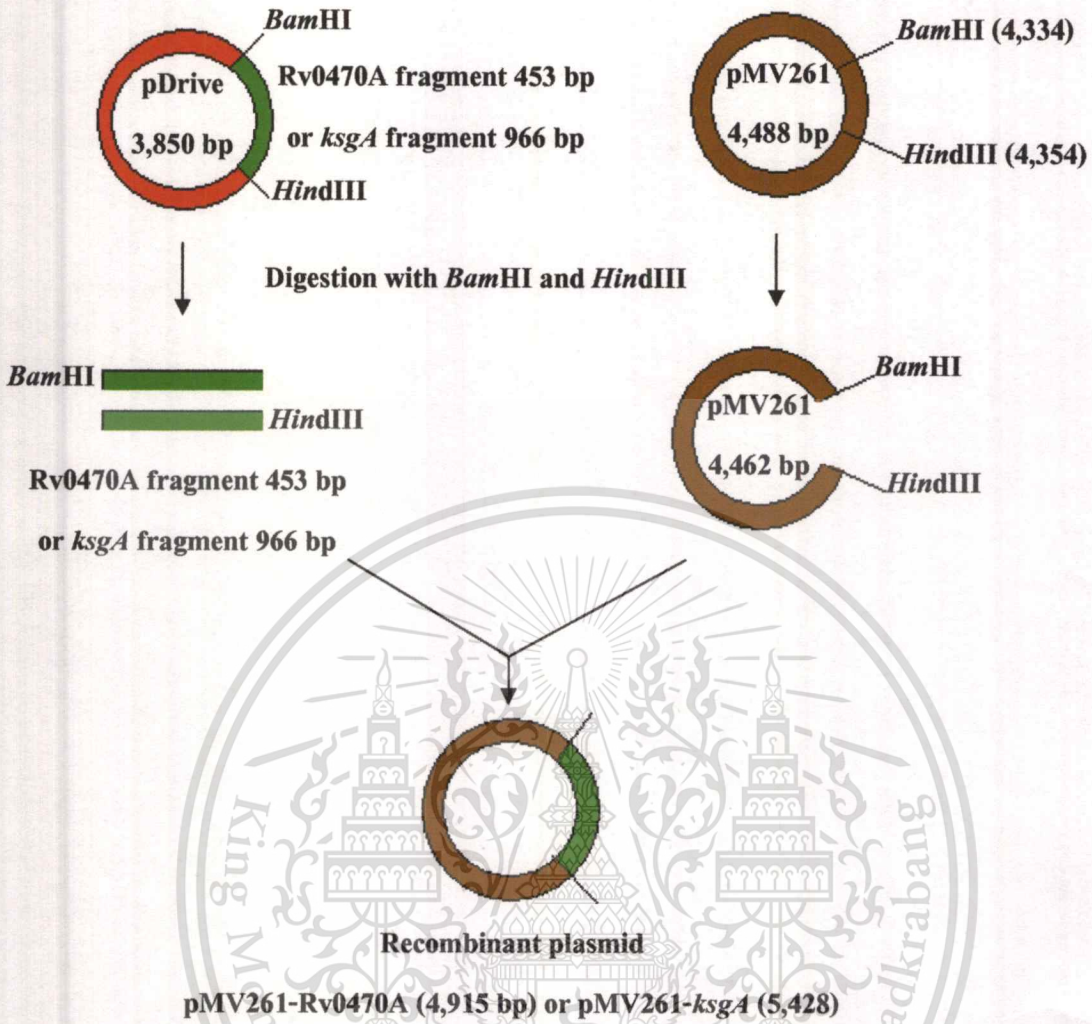


Figure 4.17 Schematic diagram showing the subcloning strategy of either Rv0470A or *ksgA* into *E.coli*/*Mycobacterium* shuttle vector pMV261

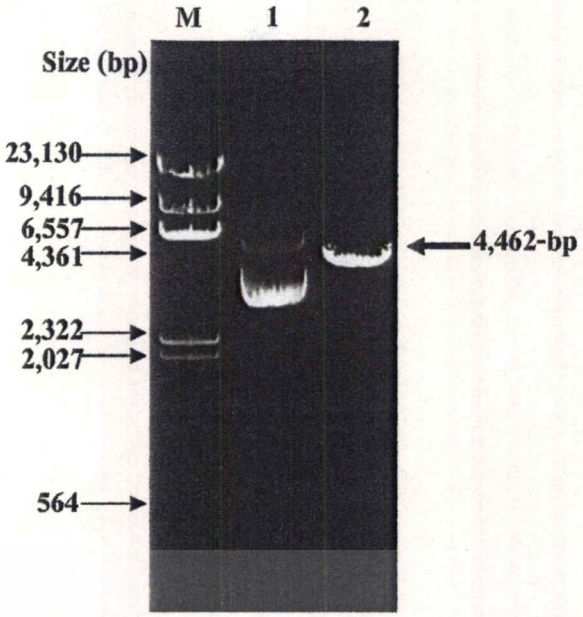


Figure 4.18 Analysis of *Bam*HI and *Hin*dIII digestion of pMV261 using 0.8% agarose gel

Lane M : λ DNA/*Hin*dII fragments

Lane 1 : Undigested pMV261

Lane 2 : *Bam*HI-*Hin*dIII digested pMV261

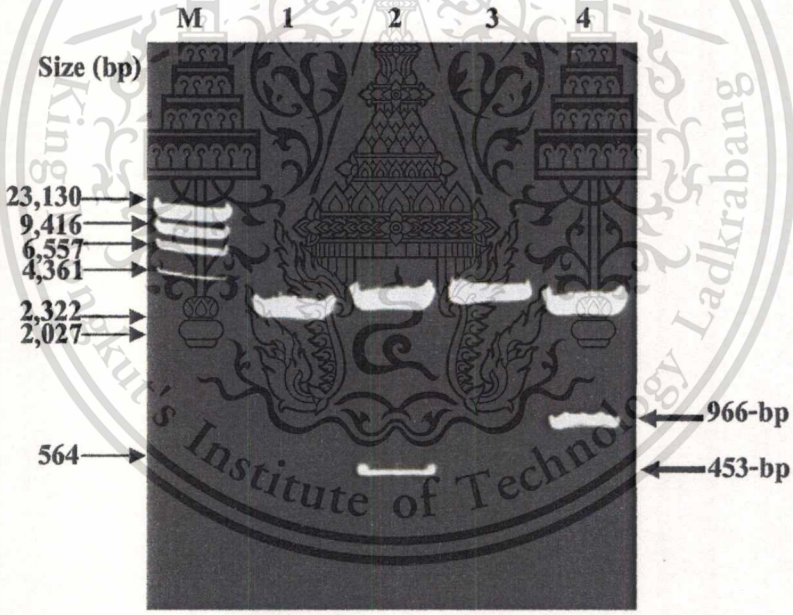


Figure 4.19 Analysis of *Bam*HI and *Hin*dIII digestion of plasmid pDrive-Rv0470A and pDrive-*ksgA* using 0.8% agarose gel

Lane M : λ DNA/*Hin*dII fragments

Lane 1 : Undigested pDrive-Rv0470A

Lane 2 : *Bam*HI-*Hin*dIII digested pDrive-Rv0470A

Lane 3 : Undigested pDrive-*ksgA*

Lane 4 : *Bam*HI-*Hin*dIII digested pDrive-*ksgA*

The presence of either Rv0470A or *ksgA* DNA fragment in the transformants was pre-screened by PCR. Crude DNA of each transformant was isolated and used as template DNA in a PCR reaction using primers pMV261-FBam and pMV261-Rcla. The PCR reaction and conditions were described in section 3.5.5.4. PCR products were analyzed using 0.8% agarose gel electrophoresis. For correct pMV261 carrying Rv0470A clone, it is expected that PCR product size should be 560 bp, consisting of 453-bp Rv0470A and 107-bp pMV261 backbone. Result revealed the expected 560-bp PCR product for transformant clones 1.2, 1.3, 1.5 and 1.6 as shown in Fig. 4.20.

Figure 4.20 Agarose gel analysis of specific 560-bp PCR product from candidate pMV261-Rv0470A clones 1.1-1.8 (lanes 1-8) using primer pMV261-FBam and pMV261-RCla (M = λ DNA/*Hin*dIII fragments)

For correct pMV261 carrying *ksgA* clone, PCR should produce 1,073-bp product, consisting of 966 bp of *ksgA* and 107 bp of plasmid backbone. As a result, expected PCR products were found from transformant clones 1.1 and 1.8 as shown in Fig. 4.21.

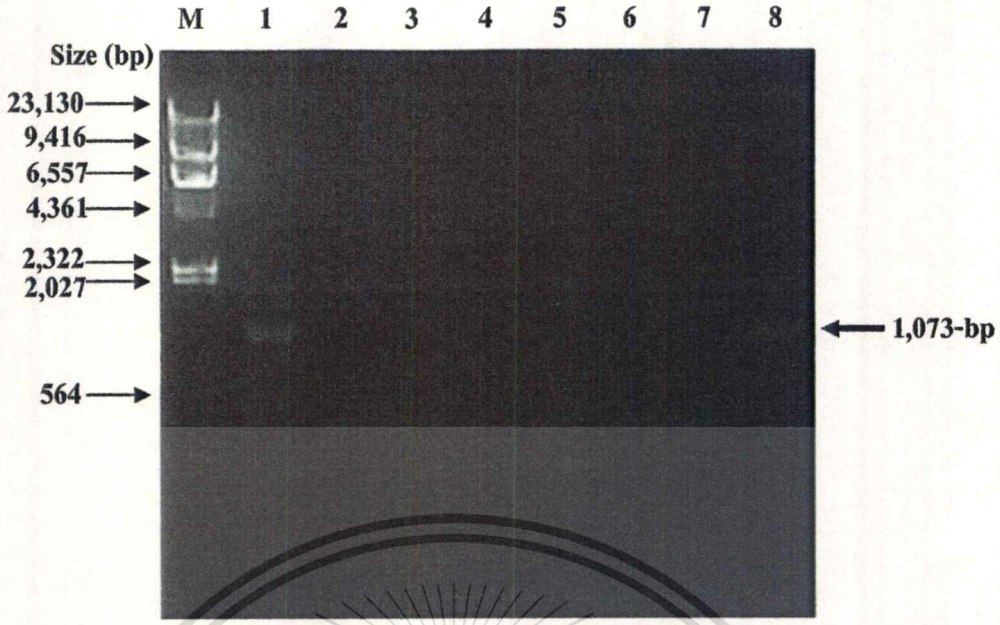


Figure 4.21 Agarose gel analysis of specific 1,073-bp PCR product obtained from candidate pMV261-*ksgA* transformant clones 1.1-1.8 (lanes 1-8) using primer pMV261-FBam and pMV261-RCla (M = λ DNA/*Hin*dIII fragments)

To be confirmed, the plasmid characterizations by several restriction endonuclease digestions were performed. Plasmid DNAs from pMV261-Rv0470A transformant clone 1.2, 1.3, 1.5 and 1.6 and pMV261-*ksgA* clone 1.1 and 1.8 were isolated and analyzed using 0.8% agarose gel electrophoresis. The result was shown in Fig. 4.22. The pMV261-Rv0470A transformant clone 1.2 and pMV261-*ksgA* clone 1.1 were selected for restriction endonuclease analysis using *Cla*I and *Xba*I for pMV261-Rv0470A and *Kpn*I for pMV261-*ksgA* respectively; the digestion reactions and conditions were shown in Table 3.7 (Section 3.5.10.3). As the presence of unique recognition site for *Cla*I, *Xba*I and *Kpn*I (3923) in pMV261 and a unique *Kpn*I site in *ksgA*, after digestion it should produce 4,063-bp and 852-bp digested bands and 4,588-bp and 840-bp digested bands for pMV261-Rv0470A and pMV261-*ksgA* respectively. Results demonstrated that both plasmids generated digested bands as expected (Fig. 4.23), indicating the correct recombinant plasmids.

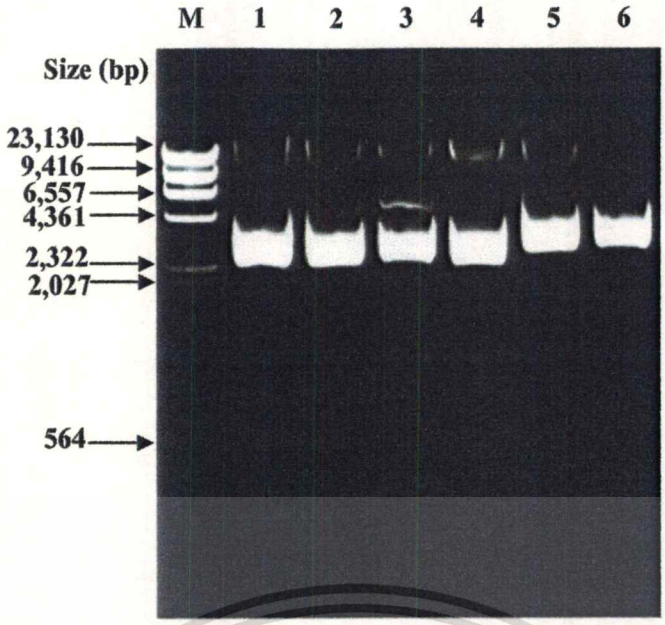


Figure 4.22 Agarose gel analysis of recombinant plasmid DNA pMV261-Rv0470A (lanes 1-4) and pMV261-*ksgA* (lanes 5-6) compared with λ DNA/*Hin*dIII fragments (lane M)

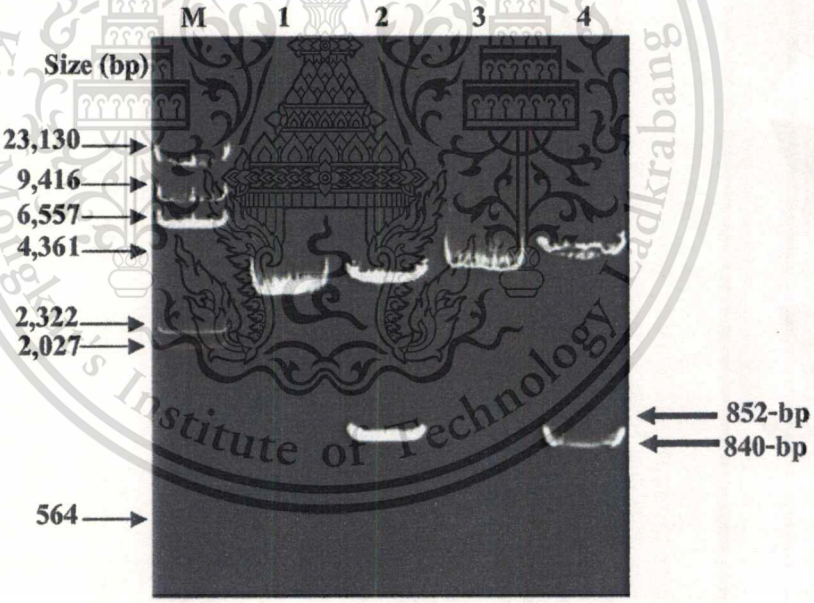


Figure 4.23 Agarose gel analysis of recombinant plasmids after restriction endonuclease digestion

Lane M : λ DNA/*Hin*dIII fragments

Lane 1 : Undigested plasmid pMV261-Rv0470A

Lane 2 : *Cla*I-*Xba*I digested pMV261-Rv0470A

Lane 3 : Undigested plasmid pMV261-*ksgA*

Lane 4 : *Kpn*I digested pMV261-*ksgA*

To confirm the correct nucleotide sequence of the cloned genes in the recombinant plasmids, both of pMV261-Rv0470A and pMV261-*ksgA* were sequenced with primers pMV261-FBam primer and pMV261-FXba primer as a sequencing primer respectively. Nucleotide sequence analysis revealed the correct nucleotide sequences of both plasmids for the cloned genes and plasmid backbone as shown in Fig. 4.24 and 4.25. Therefore, these recombinant plasmids were used to study further.

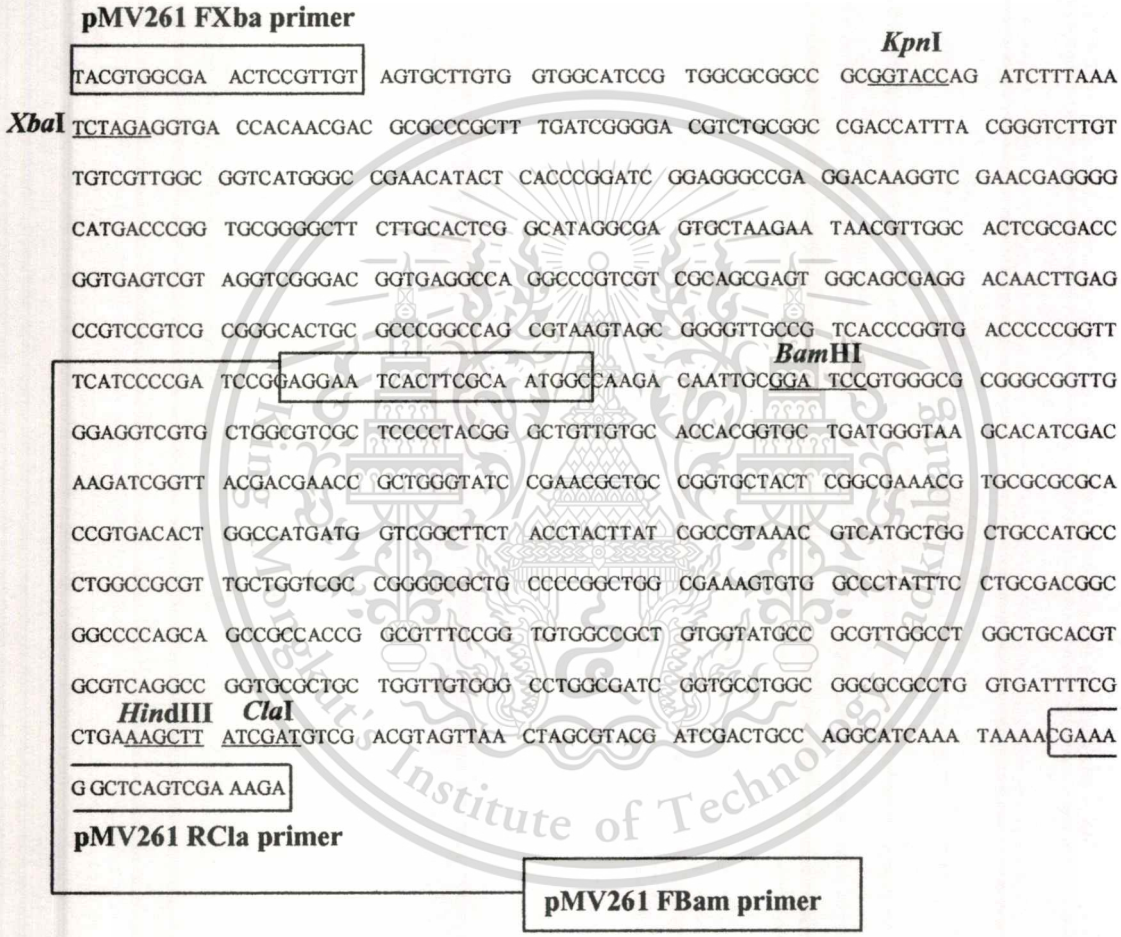


Figure 4.24 Nucleotide sequences of pMV261-Rv0470A consist of the open reading frame and plasmid pMV261, including recognition sites of *Kpn*I, *Xba*I, *Bam*HI, *Hin*dIII, and *Cla*I. Locations of primers are depicted in boxes.

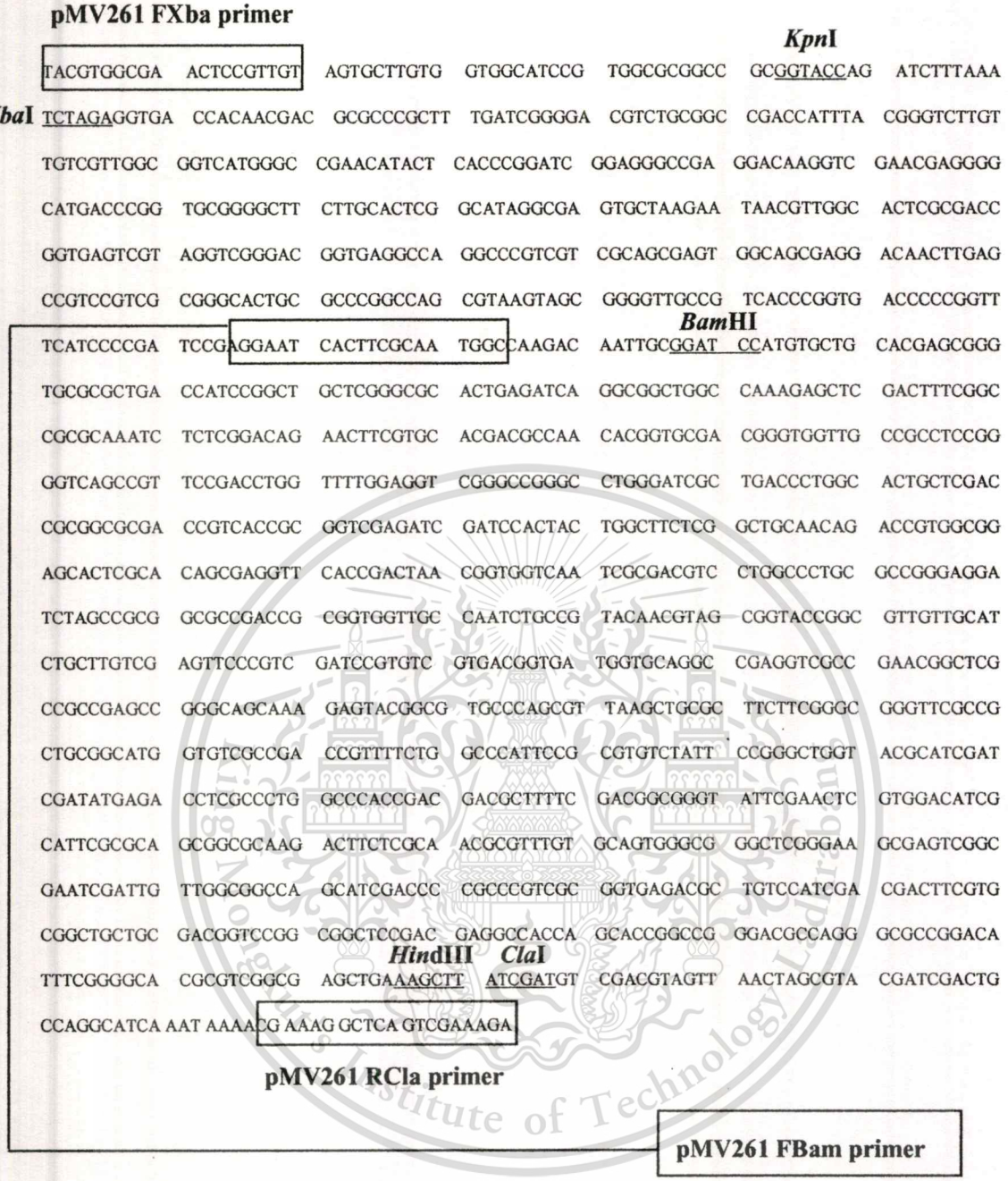


Figure 4.25 Nucleotide sequences of pMV261-*ksgA* consist of the open reading frame and plasmid pMV261, including recognition sites of *Kpn*I, *Xba*I, *Bam*HI, *Hin*dIII, and *Cla*I. Locations of primers are depicted in boxes.

4.3 Effect of overexpression of Rv0470A and *ksgA* on the clarithromycin susceptibility in *M. smegmatis*

The recombinant plasmids pMV261-Rv0470A and pMV261-*ksgA* were transformed into *M. smegmatis* mc^{2}155 by using electrotransformation. To be confirmed the presence of the desired recombinant plasmid in transformants, PCR was applied by using crude DNA from each transformant. The correct transformants were further evaluated determine the effect of overexpression of Rv0470A and *ksgA* on clarithromycin susceptibility in *M. smegmatis*.

4.3.1 Electrotransformation of pMV261-Rv0470A and pMV261-*ksgA* into *M. smegmatis* mc^2 155

Approximately 1 μg of the recombinant plasmid DNA pMV261-Rv0470A and pMV261-*ksgA* was transformed into *M. smegmatis* mc^{2}155 by electroporation as described in section 3.5.9.2. After electroporation, cells were plated on LB agar containing 25 μg/ml of kanamycin. A total 100 colonies were appeared and selected to subculture further as a single colony. One transformant from each of MSRV (*M. smegmatis* mc^{2}155 containing pMV261-Rv0470A) and MSKS (*M. smegmatis* mc^2 155 containing pMV261-*ksgA*) was cultured and crude DNA was extracted as described in section 3.5.2.2. Crude DNA was used as template for PCR by using primers pMV261-FBam and pMV261-Rcla in order to demonstrate the presence of the recombinant plasmids pMV261-Rv0470A and pMV261-*ksgA* in *M. smegmatis* mc^{2}155 transformants.

PCR results showed 560-bp and 1,073-bp DNA fragments specific to pMV261-Rv0470A and pMV261-*ksgA* respectively (Fig. 4.26). Neither parental *M. smegmatis* mc^{2}155 (MS) nor *M. smegmatis* mc^{2}155 carrying pMV261 (MS261) gave any specific PCR products (Fig. 4.26).

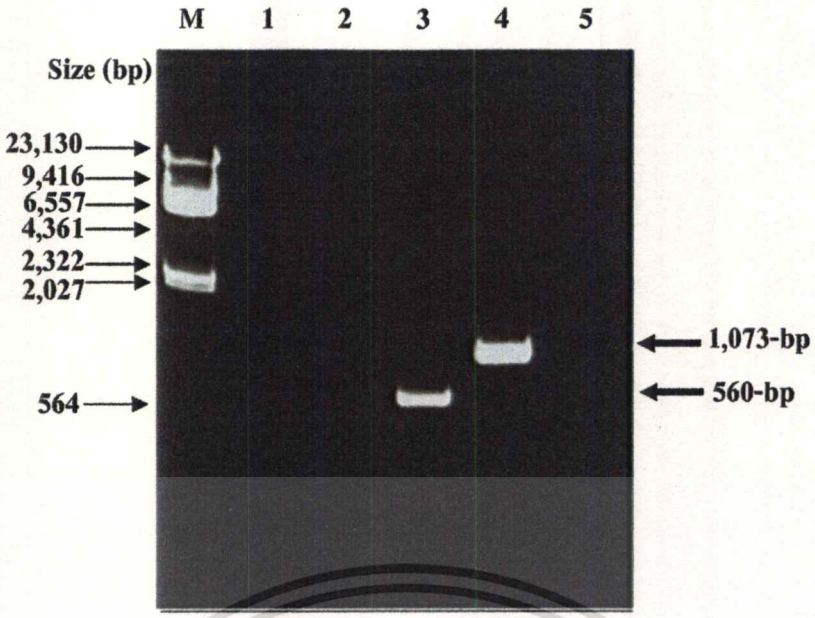


Figure 4.26 Agarose gel analysis showing specific PCR product from *M. smegmatis* transformants

Lane M : λ DNA/*Hin*dIII fragments

Lane 1 : *M. smegmatis* mc^{2}155

Lane 2 : *M. smegmatis* mc^{2}155::pMV261

Lane 3 : *M. smegmatis* mc^{2}155::pMV261-Rv0470A

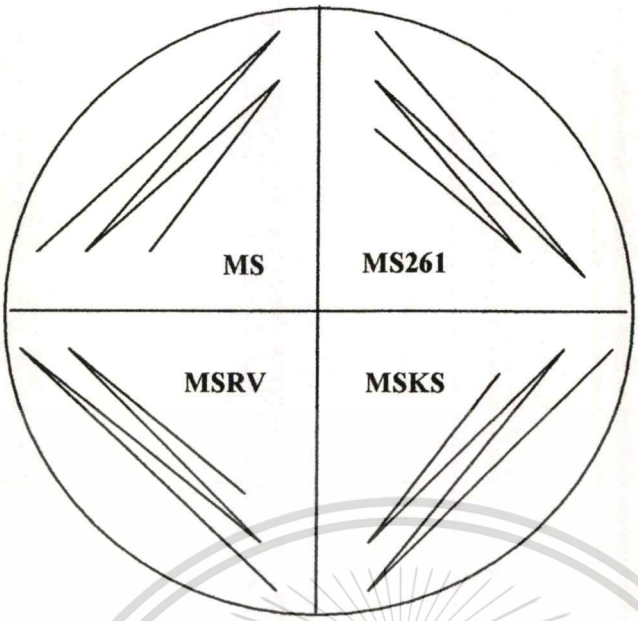
Lane 4 : *M. smegmatis* mc^{2}155::pMV261-*ksgA*

Lane 5 : Negative control (deionized water)

4.3.2 CLR susceptibility

4.3.2.1 Clarithromycin susceptibility testing of *M. smegmatis* on solid medium

CLR susceptibility test of *M. smegmatis* mc^2 155 transformants MSRV and MSKS was performed on solid medium containing 50 µg/ml of kanamycin and different concentrations of clarithromycin (0, 5, 10, 15 and 20 µg/ml) as described in section 3.5.12.1 compared to that of parental strain and *M. smegmatis* mc^2 155 carrying pMV261. Each strain was streaked in a quadrant of agar plate as shown in Fig. 4.27. Results revealed that only MSRV and MSKS were able to grow on LB plates containing 5 µg/ml of clarithromycin (Fig. 4.28). *M. smegmatis* mc^2 155 carrying pMV261 could grow only on LB and LB agar containing 50 µg/ml of kanamycin, whereas parental strain *M. smegmatis* mc^{2}155 was grown only on LB agar (Fig. 4.28). Neither *M. smegmatis* mc^2 155 transformants nor control strains could grow on LB agar containing 10, 15, and 20 µg/ml of clarithromycin (Fig. 4.28).



MS = *M. smegmatis* mc^{2}155

MS261 = *M. smegmatis* mc^{2}155::pMV261

MSRV = *M. smegmatis* mc^{2}155::pMV261-Rv0470A

MSKS = *M. smegmatis* mc^{2}155::pMV261-*ksgA*

Figure 4.27 Diagram of clarithromycin susceptibility testing of *M. smegmatis* on solid medium, the position of each strain was shown in a quadrant

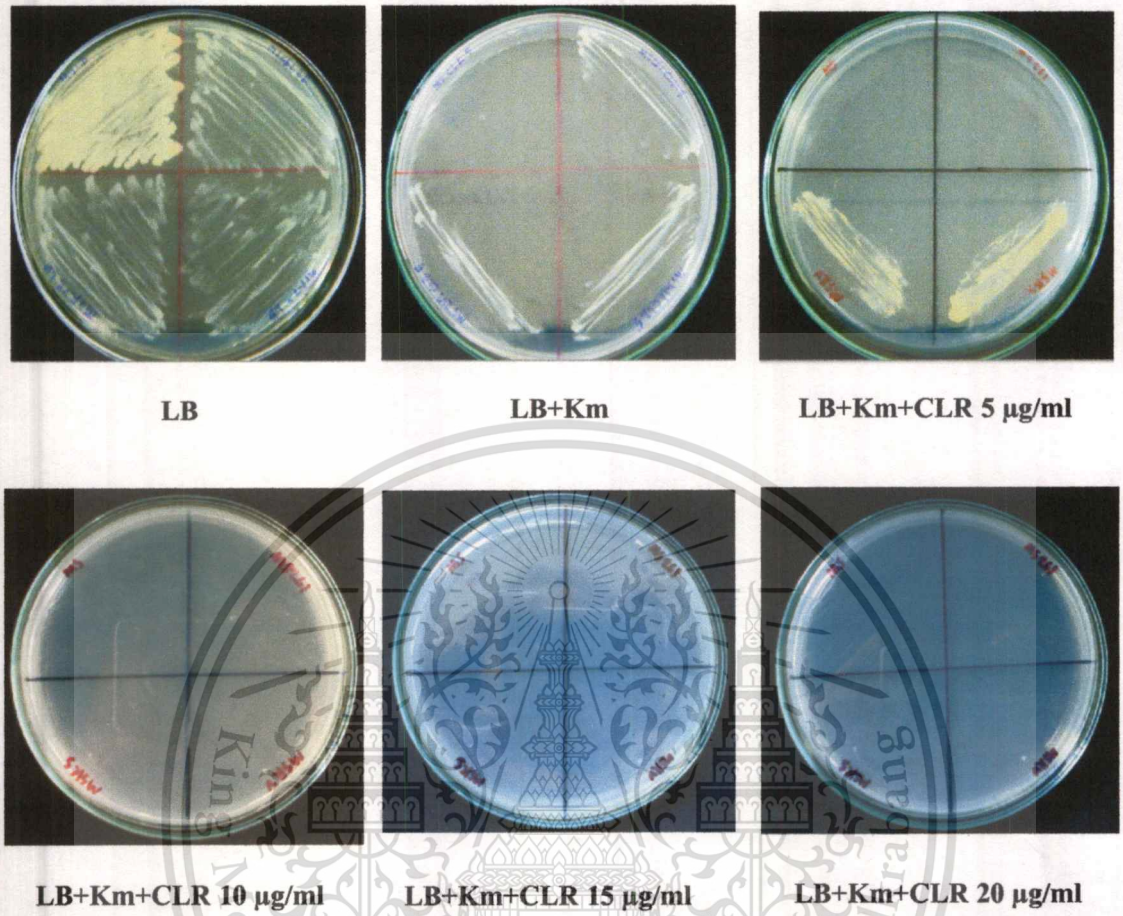


Figure 4.28 Growth of *M. smegmatis* carrying recombinant plasmid pMV261-Rv0470A (MSRV) and pMV261-*ksgA* (MSKS) on LB agar (LB), LB agar containing 50 µg/ml of kanamycin (LB+Km), LB agar containing 50 µg/ml of kanamycin and 5 µg/ml of clarithromycin (LB+Km+CLR 5 µg/ml), LB agar containing 50 µg/ml of kanamycin and 10 µg/ml of clarithromycin (LB+Km+CLR 10 µg/ml), LB agar containing 50 µg/ml of kanamycin and 15 µg/ml of clarithromycin (LB+Km+CLR 15 µg/ml), and LB agar containing 50 µg/ml of kanamycin and 20 µg/ml of clarithromycin (LB+Km+CLR 20 µg/ml), compared with *M. smegmatis* (MS) and *M. smegmatis* carrying plasmid pMV261 (MS261)

4.3.2.2 Determination of Minimum Inhibition Concentration (MIC) of CLR by the microdilution method

By using the microdilution method (as described in section 3.5.12.2), the MICs of CLR were determined. When the appearance of mycobacterial growth in control well was observed, the visible growth was also found in the other wells of Middlebrook containing clarithromycin at concentrations of 1, 2 and 4 μg/ml. Thus it suggested the lowest concentration of clarithromycin that inhibits the visible growth of *M. smegmatis* mutants MSRV and MSKS was 8 μg/ml (Table 4.3).

Table 4.3 Minimum Inhibition Concentration of clarithromycin in each strain of *M.smegmatis*

Strains	MIC of CLR (μg/ml)
MS	0
MS261	0
MSRV	8
MSKS	8

CHAPTER 5

DISCUSSION

5.1 Study of transposon localization by using genome walking technique

5.1.1 Amplification of DNA flanking to transposon Tn*5* DNA fragment

The transposon DNA fragment on genome of macrolide-susceptible *M. tuberculosis* H37Rv mutant Tn-77 and Tn-196 were identified by using genome walking approach. The procedure involves the use of amplified DNA product from the first round of PCR to serve as template for the second round PCR, whose product in turn serves as template for the third round PCR. From the first PCR reaction, the spurious PCR products were found in agarose gel electrophoresis (data not shown). Guo and Xiong (2006) suggested that the main problem encountered using degenerate primer in PCR-based genome walking is non-specific amplification of PCR products. The artificial amplification was initiated either by hybridization to the non-target locus by degenerate primer alone or by mispriming of both specific primer (TSP1 primer) and the walking primer on a non-target locus in the genomic DNA (Levano-Garcia *et al.*, 2005). However, the dense PCR product band was found in the 2nd and 3rd PCR reaction. Using different specific primers (TSP2 and TSP3) can reduce the non-specific amplification because of the spurious PCR products are unlikely to contain binding sites for the inner specific primers (Guo and Xiong, 2006).

5.1.2 Defining genes disrupted by transposon DNA fragment

The Rv0470A was annotated as a hypothetical protein with unknown function in mycobacteria (Cole *et al.*, 1998; Camus *et al.*, 2002). Amino acid sequence alignment to proteins deposited in Genbank database revealed that Rv0470A protein showed most similar to prenyl transferase family (76% identity). This transferase is localized as membrane protein and shows prenyltransferase activity associated with cell wall synthesis (Melzer and Heide, 2002; Mogi *et al.*, 1994). The mycobacterial cell wall is distinctive and is associated with the pathogenicity of *Mycobacterium tuberculosis* (Barry *et al.*, 1998). Three polymers in cell wall, arabinogalactan-mycolate (Crick *et al.*, 2001) covalently linked with peptidoglycan and trehalose dimycolate, provide a thick layer that protects the tubercle bacillus from general antibiotics. The nature of antibiotic resistance is generally attributed to the specific structure of the mycobacterial cell wall. Morris and coworkers (2005) showed the ability of *whiB7*, a putative transcriptional activator, to

mediate multiple antibiotic resistances in *Actinomycetes* and suggested that in mycobacterial species it acts synergistically with a rather impermeable cell envelope to provide high levels of intrinsic resistance. However, *whiB7* does not confer resistance to antiseptics, but rather to antibiotics containing specific targets (Example: erythromycin, streptomycin, etc.). The presence of these structural genes and corresponding regulatory systems in *Mycobacterium* suggests that this system provides selective advantage (Morris *et al.*, 2005). The fact that the *whiB7* regulon, including antibiotic resistance genes, can be activated by palmitic acid has important implications for mycobacterial chemotherapy. Palmitic acid has been found in mycobacterial cytosol and is considered to be a major source of carbon used by *M. tuberculosis* in the mammalian macrophage (Bloom, 1994). It is also the principle fatty acid found in animal tissues and serum. Therefore, the *whiB7* regulon may be induced when *M. tuberculosis* enters macrophages or other lipid rich cells, organs, or tissues, thereby allowing mycobacteria to more effectively resist some chemotherapeutic strategies or to shelter in specific areas of the body. From several lines of evidence, it could be hypothesized that the macrolide-susceptible phenotype of *M. tuberculosis* H37Rv mutant Tn-77, which has the inactivated Rv0470A, may cause by failure in system of impermeability in *M. tuberculosis* and result in macrolide-susceptibility. However, the role of Rv0470A in mycobacteria is still unclear and remains to be investigated.

The *rpfB* (Rv1009) encoded resuscitation promoting factor B; this protein exists in *M. tuberculosis*, which has five genes encoding Rpfs (*rpfA–rpfE*) (Mukamolova *et al.*, 2002; Cole *et al.*, 1998). These proteins encourage organisms recovered from dormancy. Mukamolova and colleagues (1998) reported that cell growth of dormant *Micrococcus luteus* cultures has been shown to be enhanced after addition of a resuscitation-promoting factor protein. Rpf proteins constitute a family of lytic transglycosylase enzymes capable of hydrolyzing the glycosidic bonds in the essential stress-bearing, shape-maintaining peptidoglycan layer (Mukamolova *et al.*, 2006). Two recently identified hydrolases, RpfB and RpfE, were hydrolases involved in mycobacterial cell division and shown to interact with *rpf* interacting protein (RipA) (encoded by *ripA*), a peptidoglycan endopeptidase (Hett *et al.*, 2007). Both RpfB and RipA localize to the septa of dividing bacteria (Hett *et al.*, 2007) and thus may play a role in the late stages of mycobacterial cell division. Whether none of the *rpf* genes appears to be essential in *M. tuberculosis* because bacteria encode a number of hydrolytic enzymes that are, at least in part, functionally redundant but in *M. smegmatis* and *M. tuberculosis*, *ripA* does not appear to be redundant. Strains carrying deletions of single hydrolase gene are generally viable, though combinations of mutations can

result in lack of viability (Heidrich *et al.*, 2002). One single deletion (*rpfB*) and several of the triple combinations yielded strains unable to grow or divide in stressful conditions *in vitro* and *in vivo* (Downing *et al.*, 2005; Tufariello *et al.*, 2006). This suggests that certain potential cell division proteins that perform nonessential roles in homeostatic processes can become vital in conditions of stress. In addition, Tufariello and his groups (2006) suggested that RpfB is required for resuscitation of *M. tuberculosis* in a reactivation mouse model. However, association of *rpfB* mutant and drug resistance, particularly macrolide resistance, has never been reported and should be investigated further, thus, susceptibility to macrolide of Tn-196 mutant might not involve with this gene. As a result, we focus on *ksgA* (Rv1010), kasugamycin resistance gene, which is located downstream of *rpfB* and also disrupted by the transposon.

KsgA, encoded from *ksgA*, catalyzes the transfer of methyl groups from S-adenosylmethionine to two adjacent adenosine bases, A1518 and A1519, to produce N6, N6-dimethyladenosine in 16S rRNA (Ochi *et al.*, 2009). The KsgA is homologous to another family of RNA methyltransferases, Erm, the members of which methylate a single adenosine base in 23S rRNA and confer resistance to the macrolide antibiotics (Roberts *et al.*, 1999). More than 60 Erm proteins, which can be classified into 30 different classes, have been identified to date (Roberts *et al.*, 1999; Morris *et al.*, 2005). Erm dimethylation is one of the major mechanisms of macrolide, lincosamide, streptogramin B resistance in pathogenic bacteria. The mycobacterial ErmMT confers macrolide resistance as a result of target modification, making the *M. tuberculosis* ribosomes refractory to macrolides. The mechanism action of macrolides is inhibition of bacterial protein biosynthesis, and they are thought to do this by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid as inhibiting ribosomal translocation. Another potential mechanism is premature dissociation of the peptidyl-tRNA from the ribosome (Hansen *et al.*, 2002). Kirillov and coworkers (1997) showed the binding in the polypeptide exit tunnel adjacent to the peptidyl transferase center of 15-membered macrolide, azithromycin. Their location suggests that they inhibit protein synthesis by blocking the egress of nascent polypeptides. Many resistant isolates contain enzymes that monomethylate or dimethylate the N6 position of *E. coli* A2058 in 23S rRNA, a modification that greatly reduces the affinity of ribosomes for all macrolides (Lai and Weisblum, 1971; Douthwaite, 1992; Meier *et al.*, 1994). These suggest that *E. coli* A2058 and other nucleotides belonging to the central loop of domain V in 23S rRNA are important constituents of the macrolide binding site, and imply that all macrolides bind to the same site. Macrolide resistance resulting from base changes at other

positions in the central loop of domain V of 23S rRNA (Ettayebi *et al.*, 1985; Harris *et al.*, 1989; Lucier *et al.*, 1995) are explained by the observation that such mutations alter residues that directly contact the lactone ring and thereby change the shape of the binding site.

However, there was a strong similarity of crystal structure between Erm and KsgA protein (O'Farrell *et al.*, 2004), but no methylation of 23S rRNA by KsgA have been reported. Several studies indicated the function of KsgA involved in methylation of 16S rRNA (Helser *et al.*, 1971; Helser *et al.*, 1972; Inoue *et al.*, 2007; Ochi *et al.*, 2009). Significance of this gene in bacteria is the possed functions in rRNA methyltransferase activity. Some other functions, however, have been reported in *E. coli* as a suppressor for cold-sensitive cell growth and an inducer for cell-sensitivity to extreme acid condition (Inoue *et al.*, 2007). Mutations in *ksgA* have been reported to confer resistance to kasugamycin (Vila-Sanjurjo *et al.*, 1999; Ochi *et al.*, 2009; Helser *et al.*, 1971). Although the collective importance of the rRNA modifications for protein synthesis has been demonstrated (Green and Noller, 1999), the functions of individual methylations are still unclear. The yeast KsgA ortholog (Dim1) is essential for cell growth; however, its essentiality is not due to its methyltransferase activity but depends on its pre-18S rRNA processing activity (Lafontaine *et al.*, 1998). A human KsgA ortholog with rRNA methyltransferase activity was originally found as human mitochondrial transcriptional factor B1 (Cotney and Shadel, 2006; McCulloch *et al.*, 2002; Seidel-Rogol *et al.*, 2003). A KsgA ortholog in *Arabidopsis thaliana* (Pfc1) plays an important role for chloroplast biogenesis at low temperatures (Tokuhisa *et al.*, 1998). These observations suggest that in addition to the methyltransferase activity, KsgA orthologs are recruited to play additional roles within the cells (O'farrell *et al.*, 2006), although such an additional function has not been reported for *Mycobacterium* KsgA. As mentioned above, methylation of 23S rRNA by ErmMT or *erm* (37) has been shown to mediate intrinsic macrolide resistance in *M. tuberculosis*, however, the role of other methyltransferases involved in drug resistance has not been defined in *M. tuberculosis*. Combined with the evidence from this study showing macrolide susceptible in *ksgA* mutant strain, it was hypothesized that KsgA may play a direct or, at least, an indirect role in macrolide resistance in *M. tuberculosis*. Further study should be done to clarify the additional role of KsgA in mycobacteria.

5.2 Effect of overexpressed genes on clarithromycin susceptibility in *M. smegmatis*

Homologues of Rv0470A and *ksgA* genes were found in *M. smegmatis* 51% and 92% identity respectively. Overexpression of these genes either from *M. tuberculosis* or from other mycobacteria has never been studied. In this study, overexpression of Rv0470A and *ksgA* was performed based on the fact that the replicative plasmid pMV261 would generate extrachromosomally 3-5 copies of genes per generation (Stover *et al.*, 1991). Results revealed that overexpression of either gene could increase 4-8 folds of MIC of CLR compared with the susceptible parental *M. smegmatis* strains. The reason why overexpressed genes can confer CLR resistance is still unknown.

Because of very limited information of Rv0470A, we will focus on the *ksgA*. It is possible that the *M. tuberculosis ksgA* has additional function other than methylation of 16S rRNA and may play direct or indirect role on CLR resistance. Previous studies reported that *ksgA* methylates only 16S rRNA but not 23S rRNA and overexpression of Erm increases kasugamycin sensitivity in *E. coli* (Suvorov *et al.*, 1998). In addition, study of ribosomal structure revealed that A1518 and A1519 of 16S rRNA of the 30S small ribosomal subunit and A2058 of 23S rRNA of the 50S large ribosomal subunit are located near the interface of each subunit and may interact with drug targeting in this region of both 16S and 23S rRNAs (Poehlsgaard and Douthwaite, 2005). However, all studies have never been done in mycobacteria.

It is postulated from our findings that firstly, if *ksgA* causes directly CLR resistance in *M. tuberculosis*, it should be enable to methylate A2058 of 23S rRNA, by which it can prevent CLR binding. Secondly, if it plays an indirect role, *ksgA* could not methylate A2058 but methylate A1518 and A1519 as reported in other bacteria and together with that both positions localize closely in each subunit, when they form ribosome, resulting in that methylated bases (A1518 and A1519) may interfere with CLR binding. Lack of this methylation may increase CLR sensitivity because the drug still binds to the ribosome as occurred in Tn-196 mutant strain. In order to confirm the effect of the disrupted genes on CLR susceptibility in *M. tuberculosis*, transformation of wild type Rv0470A and *ksgA* into Tn-77 and Tn-196 respectively is necessary and should be conducted next. Characterization and defining an exact role of each gene warrants to be further investigated. Nevertheles, results of the indirect functional test of either Rv0470A and *ksgA* in a surrogate host *M. smegmatis* demonstrated their roles in CLR susceptibility. This finding demonstrated a novel macrolide-resistant mechanism of *M. tuberculosis* and not only conducting to the development of new anti-TB drug but it also provides an alternative way to develop a group of macrolide for use in treatment of TB.

CHAPTER 6

CONCLUSION

This study aims to characterize two CLR-susceptible mutants of *M. tuberculosis* H37Rv, Tn-77 and Tn-196, generated from transposon mutagenesis. The insertion site of the transposon DNA fragment was identified by genome walking technique using the DNA Walking *SpeedUp*™ Premix Kit (Seegene, Korea) and DNA sequencing. The obtained sequences were aligned with the whole sequence of *M. tuberculosis* H37Rv using blastn to identify the genes inserted by the transposon DNA fragment. The disrupted genes were demonstrated whether they are associated with CLR susceptibility by overexpression the wild type genes in the CLR-susceptible *M. smegmatis* mc^2155. The wild type genes were amplified by PCR using crude DNA isolated from *M. tuberculosis* H37Rv and subcloned into the replicative plasmid pMV261 to transform and generate the overexpressing *M. smegmatis* mc^2155 mutant strains, MSRV and MSKS. The MICs of CLR for these strains were determined and compared with those of the strain harboring pMV261 alone (MS261) and the parental wild type strains.

Using the genome walking technique and sequence alignment, it was found that Tn-77 and Tn-196 had the transposon DNA fragment insertion into Rv0470A and the overlapping region of *rpfB-ksgA* respectively. Rv0470A encodes the hypothetical protein with unknown function, whereas *ksgA* codes for dimethyladenosine transferase that methylates 16S rRNA in the ribosome maturation process. Overexpression of each gene using the replicative plasmid pMV261 in *M. smegmatis* mc^2155 confers CLR resistance. MIC of CLR from the overexpressing strains was 4-8 folds higher than that of the parental *M. smegmatis* mc^2155 strain.

Although certain roles of these two genes on CLR susceptibility have not been defined, results obtained from this study were claimed to be the first study demonstrating the novel genes of *M. tuberculosis* H37Rv associated with CLR resistance mechanism, which have never been reported elsewhere. Rv0470A and *ksgA* may play the direct role or, at least, indirect role on CLR resistance. Intensive study in *M. tuberculosis* should be performed to explain how these genes involve in resistance. The outcome will provide valuable information, which could be useful for modifying or developing macrolide antibiotics and their derivatives for using against tuberculosis.

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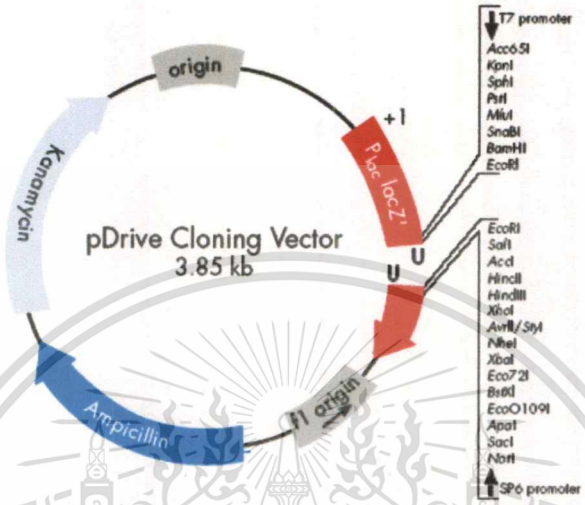
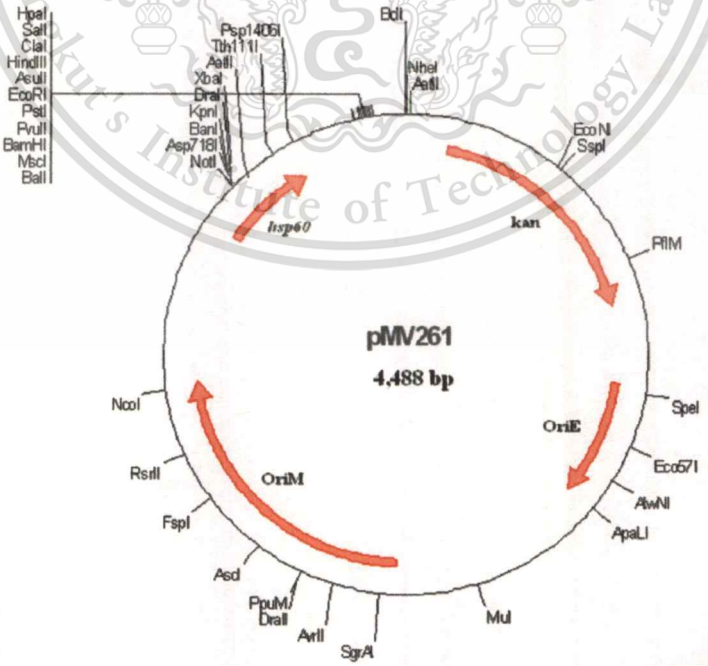
APPENDIX A

1. Mapping gene of TA cloning vector pDrive (Qiagen, Hilden, Germany)



2. Mapping gene of Mycobacterium shuttle vector pMV261

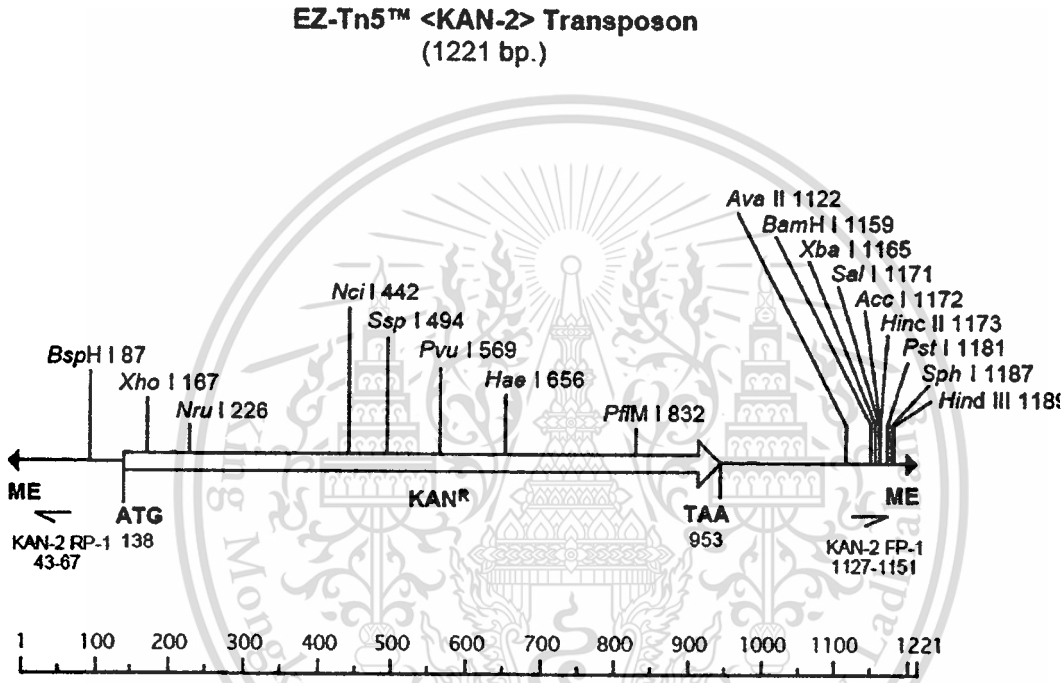


Available: http://www.einstein.yu.edu/tbresearch/Resources/Vectors/pMV261/261.jpg

APPENDIX A (CONTINUED)

3. Transposon information

3.1 *EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit* (Cat. No. TSM99K2) from Epicentre, Wisconsin, USA



Note: Not all restriction enzymes that cut only once are indicated above.
See the following pages for further information.
Primers are not drawn to scale.

KAN-2 FP-1 Forward Primer	5' ACCTACAACAAAGCTCTCATCAACC 3'
KAN-2 RP-1 Reverse Primer	5' GCAATGTAACATCAGAGATTTTGAG 3'
ME = Mosaic End	5' AGATGTGTATAAGAGACAG 3'

Available: http://www.epicentre.com/technical.htm.

APPENDIX A (CONTINUED)

3.2 Transposon nucleotide sequence

EZ-Tn5™ <KAN-2> Transposon 1221 bp.

```
   1 CTGTCTCTTA TACACATCTC AACCATCATC GATGAATTGT GTCTCAAAAT CTCTGATGTT ACATTGCACA
  71 AGATAAAAAT ATATCATCAT GAACAATAAA ACTGTCTGCT TACATAAACA GTAATACAAG GGGTGTTATG
 141 AGCCATATTC AACGGGAAAC GTCTTGCTCG AGGCCGCGAT TAAATTCCAA CATGGATGCT GATTTATATG
 211 GGTATAAATG GGCTCGCGAT AATGTCGGGC AATCAGGTGC GACAATCTAT CGATTGTATG GGAAGCCCGA
 281 TGCGCCAGAG TTGTTTCTGA AACATGGCAA AGGTAGCGTT GCCAATGATG TTACAGATGA GATGGTCAGA
 351 CTAAACTGGC TGACGGAATT TATGCCTCTT CCGACCATCA AGCATTTTAT CCGTACTCCT GATGATGCAT
 421 GGTTACTCAC CACTGCGATC CCCGGAAAAA CAGCATTCCA GGTATTAGAA GAATATCCTG ATTCAGGTGA
 491 AAATATTGTT GATGCGCTGG CAGTGTTCCT GCGCCGGTTG CATTCGATTC CTGTTTGTAA TTGTCCTTTT
 561 AACAGCGATC GCGTATTTCG TCTCGCTCAG GCGCAATCAC GAATGAATAA CGGTTTGGTT GATGCGAGTG
 631 ATTTTGATGA CGAGCGTAAT GGCTGGCCTG TTGAACAAGT CTGGAAAGAA ATGCATAAAC TTTTGCCATT
 701 CTCACCGGAT TCAGTCGTCA CTCATGGTGA TTTCTCACTT GATAACCTTA TTTTTGACGA GGGGAAATTA
 771 ATAGGTTGTA TTGATGTTGG ACGAGTCGGA ATCGCAGACC GATACCAGGA TCTTGCCATC CTATGGAACT
 841 GCCTCGGTGA GTTTTCTCCT TCATTACAGA AACGGCTTTT TCAAAAATAT GGTATTGATA ATCCTGATAT
 911 GAATAAATTG CAGTTTCATT TGATGCTCGA TGAGTTTTTC TAATCAGAAT TGGTTAATTG GTTGTAACAC
 981 TGGCAGAGCA TTACGCTGAC TTGACGGGAC GGCGGCTTTG TTGAATAAAT CGAACTTTTG CTGAGTTGAA
1051 GGATCAGATC ACGCATCTTC CCGACAACGC AGACCGTTCC GTGGCAAAGC AAAAGTTCAA AATCACCAAC
1121 TGGTCCACCT ACAACAAAGC TCTCATCAAC CGTGGCGGGG ATCCTCTAGA GTCGACCTGC AGGCATGCAA
1191 GCTTCAGGGT TGAGATGTGT ATAAGAGACA G
```

Available: http://www.epicentre.com/technical.htm.

APPENDIX B

1. Luria-Bertani (LB) medium

LB broth

Ingredients (g/L)

Tryptone	10
Yeast extract	5
Sodium chloride	10

LB agar

Ingredients (g/L)

Tryptone	10
Yeast extract	5
Sodium chloride	10
Agar	15

The pH was adjusted to 7.4 and sterilized

2. SOB medium

Ingredients (g/L)

Tryptone	20.0
Yeast extract	5.0
Sodium chloride	0.5

The pH was adjusted to 7.5 and sterilized

APPENDIX B (CONTINUED)

3. OADC (Oleic acid-albumin-dextrose-catalase)

Ingredients (g/L)

Bovine albumin fraction V	50.0
Glucose	20.0
Sodium chloride	8.5
Oleic acid	0.5
Catalase	0.04

Filter sterilized

4. Middlebrook 7H10 medium

Ingredients (g/L)

Ammonium sulfate	0.50
L-Glutamic acid	0.50
Monopotassium phosphate	1.50
Disodium phosphate	1.50
Sodium citrate	0.40
Ferric ammonium citrate	0.40
Magnesium sulfate	0.025
Calcium chloride	0.0005
Zinc sulfate	0.001
Copper sulfate	0.001
Pyridoxine hydrochloride	0.001
Biotin	0.0005
Malachite green	0.00025
Agar	15.00

APPENDIX B (CONTINUED)

5. RF1 solution

Ingredients in 1 L

KCl	10 mM
$MnCl_2 . 4H_2O$	50 mM
CH_3COOK	30 mM
$CaCl_2$	10 mM
Glycerol	15% (w/v)

The pH was adjusted to 5.8 by glacial acetic and Filter sterilized

6. RF2 solution

Ingredients in 1 L

3-N-MorpholinoXpropanesulfonic acid	10 mM
KCl	10 mM
CaCl2	10 mM
Glycerol	15% (w/v)

The pH was adjusted to 6.8 by NaOH and Filter sterilized

AUTHOR'S BIOGRAPHY

Rungaroon Suksamran was born in Bangkok, Thailand, on August 27, 1984. From 2000-2003, she studied high school at Benchamaratrangsarit Chachoengsao School in science and mathematic program. She attened the King Mongkut's Institute Technology of Ladkrabang (KMITL) in 2003, and graduated Bachelor of Science degree in Biotechnology in 2007. After that, she continued her Master education in Biotechnology at the same institute and graduated in 2010.