คุณลักษณะของยีนดื้อยากลุ่มอะมิโนไกลโคไซด์ในเชื้อวัณโรคสายพันธุ์ที่แยกได้จากผู้ป่วยและบทบาทของยีนขับยาออกนอกเซลล์ต่อการดื้อยากานามัยซินและอะมิกาซิน

CHARACTERIZATION OF AMINOGLYCOSIDE-RESISTANT GENES IN *Mycobacterium tuberculosis* CLINICAL STRAINS AND ROLE OF DRUG EFFLUX PUMP GENES ON KANAMYCIN AND AMIKACIN RESISTANCE

อังคนางค์ โสวจัสสตากุล

ANGKANANG SOWAJASSATAKUL

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
ภาควิชาชีววิทยา คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
พ.ศ. 2561

KMITL-2018-SC-M-020-034

คุณลักษณะของยีนดื้อยากลุ่มอะมิโนไกลโคไซด์ในเชื้อวัณโรคสายพันธุ์ที่แยกได้จากผู้ป่วยและบทบาทของยีนขับยาออกนอกเซลล์ต่อการดื้อยากานามัยซินและอะมิกาซิน

CHARACTERIZATION OF AMINOGLYCOSIDE-RESISTANT GENES IN *Mycobacterium tuberculosis* CLINICAL STRAINS AND ROLE OF DRUG EFFLUX PUMP GENES ON KANAMYCIN AND AMIKACIN RESISTANCE

อังคนางค์ โสวจัสสตากุล

ANGKANANG SOWAJASSATAKUL

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
ภาควิชาชีววิทยา คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
พ.ศ. 2561

KMITL-2018-SC-M-020-034

CHARACTERIZATION OF AMINOGLYCOSIDE-RESISTANT GENES IN *Mycobacterium tuberculosis* CLINICAL STRAINS AND ROLE OF DRUG EFFLUX PUMP GENES ON KANAMYCIN AND AMIKACIN RESISTANCE

ANGKANANG SOWAJASSATAKUL

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

DEPARTMENT OF BIOLOGY

FACULTY OF SCIENCE

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2018

KMITL-2018-SC-M-020-034

คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
ใบรับรองวิทยานิพนธ์

หัวข้อวิทยานิพนธ์ คุณลักษณะของยีนดื้อยากลุ่มอะมิโนไกลโคไซด์ในเชื้อวัณโรคสายพันธุ์ที่แยกได้จากผู้ป่วยและบทบาทของยีนขับยาออกนอกเซลล์ต่อการดื้อยากานามัยซินและอะมิกาซิน
Characterization of aminoglycoside-resistant genes in *Mycobacterium tuberculosis* clinical strains and role of drug efflux pump genes on kanamycin and amikacin resistance

นักศึกษา นางสาวอังคนางค์ โสวจัสสตากุล
รหัสประจำตัว 53650402
ปริญญา ปรัชญาดุษฎีบัณฑิต
สาขาวิชา เทคโนโลยีชีวภาพ
อาจารย์ที่ปรึกษาวิทยานิพนธ์ ผู้ช่วยศาสตราจารย์ ดร.สรัญญา พันธุ์พฤกษ์
อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม ดร.เทอดศักดิ์ พราหมณะนันทน์

คณะกรรมการสอบวิทยานิพนธ์	ลายมือชื่อ
รองศาสตราจารย์ ดร.อังคณา ฉายประเสริฐ ประธานกรรมการ	
ผู้ช่วยศาสตราจารย์ ดร.พนา โลหะทรัพย์ทวี กรรมการ	
ผู้ช่วยศาสตราจารย์ ดร.วรกฤต วรนันทกิจ กรรมการ	
ผู้ช่วยศาสตราจารย์ ดร.โชคชัย กิตติวงศ์วัฒนา กรรมการ	
ดร.เทอดศักดิ์ พราหมณะนันทน์ กรรมการ	
ผู้ช่วยศาสตราจารย์ ดร.สรัญญา พันธุ์พฤกษ์ กรรมการ	

วัน / เดือน/ ปี ที่สอบ วันอังคารที่ 3 กรกฎาคม พ.ศ. 2561 เวลา 13.00-16.00 น.
สถานที่สอบ ณ ห้อง 439 อาคารจุฬาภรณวลัยลักษณ์ 1

คณะวิทยาศาสตร์รับรองแล้ว

(รองศาสตราจารย์ ดร.อิทธิพล แจ้งชัด)
คณบดีคณะวิทยาศาสตร์
วันที่ 20 **เดือน** กรกฎาคม **พ.ศ.** 2561

หัวข้อวิทยานิพนธ์	คุณลักษณะของยีนดื้อยากลุ่มอะมิโนไกลโคไซด์ในเชื้อวัณโรคสายพันธุ์ที่แยกได้จากผู้ป่วยและบทบาทของยีนขับยาออกนอกเซลล์ต่อการดื้อยากานามัยซินและอะมิกาซิน
ชื่อนักศึกษา	นางสาวอังคนางค์ โสวจัสสตากุล
รหัสประจำตัว	53650402
ปริญญา	ปรัชญาดุษฎีบัณฑิต
ภาควิชา	ชีววิทยา
พ.ศ.	2561
อาจารย์ที่ปรึกษาวิทยานิพนธ์	ผศ.ดร.สรัญญา พันธุ์พฤกษ์
อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม	ดร.เทอดศักดิ์ พราหมณะนันทน์

บทคัดย่อ

วัณโรคเป็นโรคติดเชื้อในระบบทางเดินหายใจที่เกิดจากการติดเชื้อแบคทีเรีย *Mycobacterium tuberculosis* อุบัติการณ์ของวัณโรคดื้อยาหลายขนาน (multidrug-resistant tuberculosis; MDR-TB) และวัณโรคดื้อยาทุกขนานชนิดรุนแรง (extensively drug-resistant tuberculosis; XDR-TB) เป็นปัญหาทางด้านสาธารณสุขทั่วโลก ดังนั้น จึงมีความพยายามมุ่งเน้นศึกษากลไกการดื้อยาของเชื้อวัณโรค รวมถึงการพัฒนายาต้านวัณโรคชนิดใหม่ ในงานวิจัยนี้ ได้ทำการศึกษาลำดับนิวคลีโอไทด์ของยีนที่เกี่ยวข้องกับการดื้อยากลุ่มอะมิโนไกลโคไซด์ทั้งหมด 5 ยีน ได้แก่ ยีน *rrs* บริเวณโปรโมเตอร์ของยีน *eis* ยีน *tap* ยีน *whiB7* และยีน *tlyA* ในเชื้อวัณโรคที่ดื้อยากานามัยซินจำนวน 29 สายพันธุ์และเชื้อวัณโรคไวต่อยากานามัยซินจำนวน 27 สายพันธุ์ ที่คัดแยกจากผู้ป่วยวัณโรคในประเทศไทย งานวิจัยนี้แสดงให้เห็นว่า การดื้อยากานามัยซินของเชื้อวัณโรคที่พบในประเทศไทยส่วนใหญ่เกิดจากการกลายพันธุ์ที่ของยีน *rrs* หรือยีน 16S rRNA ที่ตำแหน่ง A1401G ในเชื้อวัณโรคที่ดื้อยากานามัยซินจำนวน 29 สายพันธุ์ พบการกลายพันธุ์ของยีน *rrs* ที่ตำแหน่ง A1401G จำนวน 21 สายพันธุ์ และการกลายพันธุ์ที่บริเวณโปรโมเตอร์ของยีน *eis* ที่ตำแหน่ง C-14T หรือ G-37T จำนวน 5 สายพันธุ์ นอกจากนี้ ในเชื้อดื้อยากานามัยซินจำนวน 2 สายพันธุ์ ยังพบการกลายพันธุ์ของยีน *tap* ที่ตำแหน่ง Ins581C ส่วนเชื้อดื้อยากานามัยซินอีก 1 สายพันธุ์ที่เหลือคือ *M. tuberculosis* สายพันธุ์ MT433 ไม่พบการกลายพันธุ์ของยีนที่ทำการศึกษาทั้งหมด ถึงแม้ว่าเชื้อสายพันธุ์นี้มีการดื้อต่อยาอะมิกาซินและยากานามัยซินในระดับสูง (64 ไมโครกรัมต่อมิลลิลิตร) จากการศึกษาระดับการแสดงออกของยีนที่เกี่ยวข้องกับกลไกการขับยาหรือลดการสะสมของยาอะมิโนไกลโคไซด์ภายในเซลล์ รวมทั้งยีน hypothetical transmembrane จำนวน 16 ยีนด้วยวิธี Real-time qRT-PCR พบว่าเชื้อ *M. tuberculosis* สายพันธุ์ MT433 มีระดับการแสดงออกของยีน Rv1819C, Rv1877 และ Rv2846C สูง (>2.6 เท่า) ภายใต้การกดดันของยากานามัยซินเทียบกับเชื้อ *M. tuberculosis*

สายพันธุ์ MT164 ที่มีการกลายพันธุ์ของยีน *rrs* ที่ตำแหน่ง A1401G จากการทดสอบความไวต่อยาอะมิกาซินและกานามัยซินในเชื้อ *M. tuberculosis* H37Ra ที่มีการแสดงออกที่เพิ่มสูงขึ้นของยีน Rv1819c, Rv1877 และ Rv2846c พบว่า ทั้งสามยีนนี้ไม่เกี่ยวข้องโดยตรงกับการดื้อต่อยาอะมิโนไกลโคไซด์ในเชื้อ *M. tuberculosis* สายพันธุ์ MT433 อย่างไรก็ตาม การแสดงออกของยีน *eis* ในระดับสูงในสายพันธุ์นี้อาจมีบทบาทต่อการดื้อยากลุ่มอะมิโนไกลโคไซด์

คำสำคัญ : วัณโรค *Mycobacterium tuberculosis* อะมิโนไกลโคไซด์ อะมิกาซิน กานามัยซิน การขับยาออกนอกเซลล์

Thesis Title	Characterization of aminoglycoside-resistant genes in *Mycobacterium tuberculosis* clinical strains and role of drug efflux pump genes on kanamycin and amikacin resistance
Student Name	Miss Angkanang Sowajassatakul
Student ID	53650402
Degree	Doctor of Philosophy
Department	Biology
Year	2018
Thesis Advisor	Asst. Prof. Dr. Saranya Phunpruch
Thesis Co-advisor	Dr. Therdsak Prammananan

Abstract

Tuberculosis (TB) is an infectious respiratory disease caused by an infection of *Mycobacterium tuberculosis*. The emergency of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) is worldwide public health problem. Therefore, many efforts have focused on studying drug resistance mechanisms and the development of new antituberculosis drugs. In this study, genes associated with aminoglycoside resistance including *rrs*, *eis* promoter region, *tap*, *whiB7* and *tlyA* were investigated in twenty-nine kanamycin-resistant *M. tuberculosis* clinical strains isolated in Thailand and twenty-seven kanamycin-susceptible strains. This study demonstrated that the majority of kanamycin resistance in Thai *M. tuberculosis* clinical strains was *rrs* or 16S rRNA mutation at A1401G. Mutation of *rrs* at A1401G was found in twenty-one out of twenty-nine kanamycin-resistant strains whereas mutations of *eis* at C-14T or G-37T were found in the remaining five kanamycin-resistant strains. The other two remaining kanamycin-resistant strains showed Ins581C mutation in *tap* gene. No mutations in all kanamycin resistant genes were found in *M. tuberculosis* MT433, although it showed a high-level resistance to AMK and KM (64 μg/ml). The expression level of sixteen efflux pump or hypothetical transmembrane genes was investigated by real-time quantitative reverse transcription PCR method (Real-time qRT-PCR). The result revealed that *M. tuberculosis* MT433 showed a high-level of gene expression in Rv1819c, Rv1877 and Rv2846c (>2.6-fold

change) under kanamycin exposure compared to *M. tuberculosis* MT164 with A1401G *rrs* mutation. By AMK and KM susceptibility testing in overexpressed *M. tuberculosis* H37Ra containing Rv1819c, Rv1877 and Rv2846c, these genes was not directly associated with aminoglycoside resistance in *M. tuberculosis* MT433. However, the high-level expression of *eis* in this strain might play a role in aminoglycoside resistance.

Keywords : Tuberculosis, *Mycobacterium tuberculosis*, Aminoglycoside, Amikacin, Kanamycin, Drug efflux pump

Acknowledgements

I would like to express my sincere gratitude to my advisor Assistant Professor Dr.Saranya Phunpruch and co-advisor Dr.Therdsak Prammananan for all the continuous support of my Ph.D. study and research. I would like to thank my advisor to give me a chance and accept me as her Ph.D. student. I have been extremely lucky to have an advisor who cares and give me great consults and valuable comments. I would also like to thank my co-advisor for his helpful suggestion and excellent discussion on my entire thesis including reviewing my manuscript.

This work would not have been possible without the financial support from the Thailand Graduate Institute of Science and Technology scholarship (TGIST), National Science and Technology Development Agency (NSTDA).

My gratitude is also extended to Assistant Professor Dr.Pana Lohasupthawee, Assistant Professor Dr.Chokchai Kittiwongwattana, Assistant Professor Dr.Worakrit Worananthakij and Associate Professor Dr.Angkana Chaiprasert, for serving as thesis committee, for their valuable comments and useful suggestions.

Moreover, I would like to thank all members of staff in Drug Resistance Tuberculosis Research Fund under the Patronage to Her Royal Highness Princess Galayani Vadhana Krom Luang Naradhiwas Rajanagarindra, Faculty of Medicine, Siriraj hospital, Tuberculosis Research Laboratory (NSTDA) and molecular room at Department of Biology, King Mongkut's Institute of Technology Ladkrabang (KMITL) which supported and encouraged me. I would like to give a special thanks to Miss Namphun Makao, Miss Sarinya Jaitrong, Miss Sunisa pansook and Miss Nichanan Thinpranee for their help and suggestion.

Finally, I deeply appreciate my parent, family and everyone for their support and encouragement that make a success of my Ph.D. graduation.

Angkanang Sowajassatakul

Table of Contents

Page

Table of Contents

Page

Table of Contents

Page

Table of Contents

Page

Table of Contents

Page

List of Tables

Tables **Page**

List of Tables

Tables **Page**

List of Figures

Figure **Page**

List of Figures

Figure **Page**

List of Figures

Figure **Page**

Chapter 1
Introduction

1.1 Research motivation

Tuberculosis or TB is one of the serious bacterial infectious diseases worldwide. TB is caused by an infection of the acid-fast bacterium *Mycobacterium tuberculosis* (Mtb). It can occur in the human lungs and all parts of body. Symptoms of TB are coughing up blood, chest pain, weight loss, chills, night sweats and fever. This disease causes human mortality in children, elder, patients with malnutrition, and especially human immunodeficiency virus (HIV) infected individuals. In 2014, World Health Organization (WHO) reported that there were 1.5 million TB deaths (1.1 million HIV-negative and 0.4 million HIV-positive) all over the world and Thailand was one of the high TB burden countries (WHO, 2015).

TB is a curable disease, if the disease is rapidly and accurately diagnosed and patients obtain a proper and effective treatment regimen. In general, the first-line antituberculosis drugs, consisting of isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin, have been used to treat drug-susceptible *M. tuberculosis* strains whereas the second-line drugs, such as fluoroquinolones, aminoglycosides, capreomycin, ethionamide, para-aminosalicylic acid, and cycloserine, have been used for treatment of drug-resistant strains. The strategy for TB control recommended by WHO is DOTS (directly observed treatment, short-course) which includes a direct standardized regimen treatment for 6 months and an observation by a healthcare worker or community health worker for all courses of treatment. Resistance to antituberculosis drugs is an emergency problem resulting either from an increase in the number of HIV/AIDS patients or a mismanagement of TB drugs. Multidrug-resistant TB (MDR-TB) is caused by a strain resistant to at least two most effective first-line drugs, isoniazid and rifampicin. In addition, MDR-TB that additionally resists to any fluoroquinolones and one of three injectable second-line drugs (kanamycin, amikacin, and capreomycin) is designated as extensively drug-resistant TB (XDR-TB). Therefore, a rapid detection together with an appropriate treatment of drug-resistant

TB is urgently required for effective TB control. Basic mechanisms of drug resistance have been intensively investigated in the past few decades but, for several drugs, they are not well understood.

Aminoglycosides, such as kanamycin, amikacin, gentamicin and streptomycin, are broad-spectrum and high potential drugs for infectious disease treatment including TB. Aminoglycosides are structurally composed of aminocyclitol, ribose and amino-sugar groups, making them soluble in water. The drugs bind to a 16S rRNA of small ribosomal subunit, leading to an inhibition of protein synthesis. Three known aminoglycoside resistance mechanisms are involved in (i) an alteration of drug target, (ii) an enzymatic modification, and (iii) a drug efflux pump.

Until now, *rrs* (Rvnr01), *eis* (Rv2416c), *tap* (Rv1258c), and *whiB7* (Rv3197A) have been shown to associate with aminoglycoside resistance in *M. tuberculosis*. In this study, these genes will be characterized in kanamycin-resistant M. tuberculosis strains compared with kanamycin-susceptible strains. Identification of the resistance mechanisms, particularly a novel one, is important for the development of surrogate markers that can be combined with other known resistance determinants and can improve the sensitivity and specificity of the genetic tests for rapid detection of drug-resistant strains.

However, one clinical strain, designated as *M. tuberculosis* MT433, showed kanamycin- and amikacin-resistant phenotype but did not contain any known mutations associated with aminoglycoside resistance, indicating an unknown mechanism conferring a drug resistance. Drug efflux pump is one of the possible mechanisms that might play an important role in aminoglycoside resistance. Efflux pump is a protein transporter that extrudes the toxic substances, drugs or antibiotics out of the cells. Efflux pump proteins are classified into 5 families based on amino acid sequences and energy sources used to export (ATP or sodium gradient). They are (i) Major facilitator superfamily (MFS), (ii) ATP-binding cassette superfamily (ABC), (iii) Small multidrug resistance superfamily (SMR), (iv) Resistance-nodulation-cell division superfamily (RND), and (v) Multi antimicrobial extrusion protein superfamily (MATE). There have been many efflux pump proteins found in *M. tuberculosis*. Some have already been experimentally proven; some are hypothetical proteins that are

predicted as drug efflux proteins by bioinformatic tools. Therefore, in this study, genetic characterization of the aminoglycoside resistant genes in clinical strains and role of putative drug efflux pumps on kanamycin and amikacin resistance in the *M. tuberculosis* MT433 strain will be investigated.

1.2 Objectives of the study

1) To investigate the genes associated with aminoglycoside resistance (*rrs*, *eis* including promoter, *tap*, *tlyA*, and *whiB7*) in kanamycin-resistant *M. tuberculosis* strains isolated in Thailand.

2) To explore a role of drug efflux pump on amikacin and kanamycin resistance in *M. tuberculosis* MT433, a resistant strain with unknown mechanism, by genetic characterization and determination of expression level of drug efflux genes.

1.3 Scopes of the study

1) Genes associated with kanamycin, amikacin, and capreomycin resistance [*rrs* (Rvnr01), *eis* (Rv2416c), *tap* (Rv1258c), *whiB7* (Rv3197A) and *tlyA* (Rv1694)], and minimal inhibition concentration (MIC) of kanamycin, amikacin and capreomycin will be investigated in 29 kanamycin-resistant clinical strains (26 XDR-TB and 3 MDR-TB) isolated from patients in Thailand.

2) Nucleotide sequence and expression level of one regulator (Rv3194A), *eis* (Rv2416c) and 16 putative efflux pump genes (Rv0783c, Rv1250, Rv1258c, Rv1410c, Rv1634, Rv1877, Rv2333, Rv2846c, Rv0194, Rv1456c, Rv1457c, Rv1458c, Rv1819c, Rv1145, Rv1146 and Rv3065) will be investigated in amikacin- and kanamycin-resistant *M. tuberculosis* MT433.

3) The function of a postulated drug efflux pump gene will be proven by over-expressing the gene in *M. tuberculosis* H37Ra using the replicative plasmid pSMT1 and determining aminoglycoside susceptibility.

1.4 Benefits of the study

This research will provide more understanding of aminoglycoside resistance mechanism and demonstrate the distribution of amikacin, kanamycin, and capreomycin resistance genes among Thai pre-XDR and XDR-TB strains. This is the first study characterization of Thai aminoglycoside-resistant *M. tuberculosis* strains at the molecular level. In addition, the role of putative efflux pumps on aminoglycoside susceptibility will be experimentally demonstrated in *M. tuberculosis* MT433 (DS no. 24433).

Chapter 2

Theory and Literature Reviews

2.1 Tuberculosis

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* complex, mostly by *Mycobacterium tuberculosis* and *Mycobacterium bovis*. TB continues to cause the human mortality and morbidity, especially in the underdeveloped and developing countries (WHO, 2015). In 1882, the discovery of *M. tuberculosis* was firstly published by the German physician "Robert Koch" (Sakula, 1982). TB has been found in specific microorganisms that could be transferred from one species to others, including castle and human (Helman *et al*., 1998; Michel *et al*., 2003; Thorel *et al*., 1998). TB is an ancient disease but is still a major cause of health problem over the world. WHO reported that there were approximately 10.4 million new cases of TB and 1.4 million deaths in 2015, mostly found in developing countries. The South-East Asia is accounted for a region containing a large number of TB cases, approximately 5 million prevalent cases and 3.5 million incident cases of TB in 2015. Thailand is ranked 18th on the list of 22 TB high-burden countries, with an estimated 117,000 new incident cases of TB and 12,000 deaths in 2015 (WHO, 2015). In addition, the expanding Human Immunodeficiency Virus (HIV) epidemic is a growing concern for TB because TB is the major cause of death among Acquired Immune Deficiency Syndrome (AIDS) patients, existing approximately 16,000 incidents cases of TB with HIV-positive (Kwan *et al*., 2011).

Tuberculosis primarily infected in the lungs, which is called "Pulmonary Tuberculosis", is usually transmitted from one person to another via breathing or infectious droplets when patients cough, sneeze, shout, sing or laugh. Furthermore, the active TB can spread to the lungs or other parts of the body and later symptoms are present in a week. In addition, TB can occur outside the lung which is known as "Extrapulmonary Tuberculosis". Extrapulmonary Tuberculosis can be found in many organs which is called in a different name, for example, TB found in lymph nodes is called "Lymph node TB", TB found in bones and joints is called "Skeletal TB", TB

found in digestive system is called “Gastrointestinal TB”, TB found in bladder and reproductive system is called “Genitourinary TB” and TB found in nervous system is called “Central nervous system TB”.

The researchers in many countries are interested in TB problems and attempt to find the way to defend against TB spread. The immunology system, pathogenesis of tuberculosis, symptoms, the diagnosis of TB and drug resistance mechanisms have been extensively studied and can be summarized as following;

2.1.1 Immunology and pathogenesis of tuberculosis

An infection of TB normally occurs when persons with the pulmonary or laryngeal TB diseases cough, sneeze, shout or sing. *M. tuberculosis* is transmitted through the air to a person who inhales small airborne droplet nuclei containing these bacilli (Fig. 2.1). These infectious droplets are approximately 0.5 to 5 micrometers in diameter. About 3-4 % of infected individuals can develop an active TB disease and moreover, HIV positive patients have a higher chance of recieving TB. When *M. tuberculosis* reaches the pulmonary alveoli of lungs, they are ingested by an alveolar macrophage and TB bacilli are mostly killed or inhibited by a human immunology system after 2 to 8 weeks. The living bacilli multiply inside the cells and are released after the death of macrophage. After cell release, *M. tuberculosis* may spread via lymphatic channels and bloodstream to the other parts of body such as lymph node, abdominal cavity, pericarditis, osteal, renal, adrenal, brain and spinal cord (Herrmann *et al*., 2005).

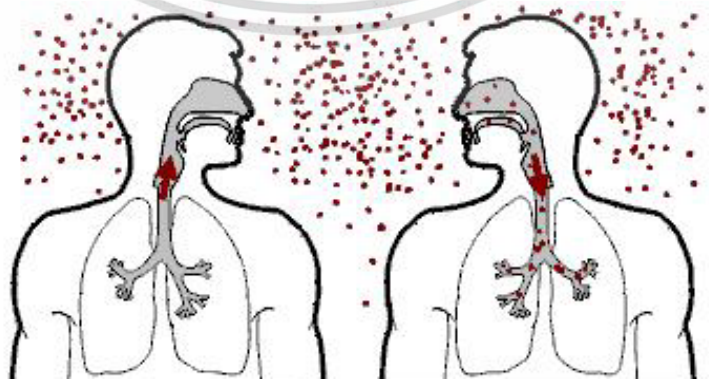


Figure 2.1 Transmission of tuberculosis in human

Available : http://www.eac.int/health/images/tb/transmission.jpg

The pathogenesis of pulmonary TB is classified into four stages (Dannenberg, 1993).

Stage 1: It is a period after 1 to 7 days of infection. The first infection of *M. tuberculosis* activates an alveolar macrophage of immunology. The bacilli are surrounded and destroyed by macrophages, resulting in an inhibition of *M. tuberculosis* growth, although some bacilli are alive in the lungs.

Stage 2: It is a period after 7 to 21 days of infection. The alive bacilli grow inside macrophages till macrophages die. After the death of macrophage, *M. tuberculosis* is released and activates monocytes in the bloodsteam. The combination of monocytes at the infection region becomes "Granuloma".

Stage 3: It is a period after three weeks of infection. The multiply of bacilli (about 10^3-10^4 bacilli) activates cellular immune response that affects cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH), resulting in the caseous necrosis. CMI is a T-cell mediated defense mechanism against survival microbes within phagocytes or infected nonphagocytic cells. In TB pathogenesis, the presence of CMI and DTH makes a solid caseous material (arresting the living bacilli) and makes activated macrophages.

Stage 4: It is a period of the presence of liquefaction and cavity in lungs tissues. This stage is one of the most intriguing aspects of human TB. Liquefaction of caseous material is a reaction between DTH and tuberculin-like product. In the liquefied caseum, virulent bacilli can multiply outside the cells, especially in the liquefied caseum next to the inner wall of lung.

Stage 1 to stage 3 is primary TB pathogenesis in patients with common immunology. Some patients with abnormal cell-mediated immunity (CMI) become worse and enter stage 4. An infection of persons who carry *M. tuberculosis* in bodies but they do not express TB disease and do not spread TB infection to other people, is called "Latent tuberculosis infection (LTBI)". In the beginning of LTBI, the extracellular bacilli are ingested by macrophages and bacilli are appeared in to other white blood cells. White blood cells kill or encapsulate the majority of bacilli, making a formation of granuloma (Gohn focus).

2.1.2 Symptoms

General symptoms of active TB are fever, weight loss, feeling very tired, fatigue, chills and night sweats. In the pulmonary TB, active symptoms begin over a period of weeks or months that are a cough with thick cloudy sputum up blood (TB gets into blood vessel in the lungs), chest pain and shortness of breath.

Although the most commonly found TB is the pulmonary TB but MTB can infect other organs or body systems. The extrapulmonary TB is often occurred in children or immunosuppressed patients. Symptoms of patients with extrapulmonary TB depend on the infected tissues or organs. The most common infected site is lymph nodes which are draining stations of lymph and contain macrophages in the body. In lymph node TB, nodes swollen in one location in the body press the surrounding structure or tissue. Some nodes are swollen around windpipe, resulting in a difficulty of breathing and stridor. The other types of extrapulmonary TB show various symptoms, for example, collapse and fracture of bones (skeletal TB), upper gastrointestinal bleeding and gastritis obstruction (gastrointestinal TB), increasing of frequency of urination and blood or pus in the urine (genitourinary TB), and lower extremity weakness or bowel or bladder (central nervous system TB). In addition, some TB patient cases do not show symptoms, therefore diagnosis processes of TB are required to confirm TB disease.

2.1.3 Diagnosis of mycobacteria

The initial step of TB evaluation is an inquiry of medical history from patients. The diagnosis of mycobacterium and *M. tuberculosis* has many various methods with a different effectiveness. It can generally be separated into 4 methods.

2.1.3.1 Conventional method

A conventional method is dependent on the physical and biochemical properties of mycobacterium. Physical properties are, for example, growth rate, temperature for growth, pigmentation and colony morphology. Primary and supplementary tests are used for biochemical tests including the tests of niacin (niacin is detected by addition of cyanogens bromide and aniline in the culture, resulting in yellow color of culture (positive)), 68 ℃ catalase test, tween hydrolysis, nitrate reduction, urease, arylsulfatase, microscopy, optimal temperature (25 ℃, 37 ℃

and 45 °C), iron uptake, pyrazinamidase, tellurite reduction, resistance to inhibitory agents (Isoniazid (INH) and thiocholine (TCH)), 5% NaCl tolerance, growth on Mac Conkey agar and serotyping. *M. tuberculosis* shows the biochemical properties different from other mycobacteria. It displays positive niacin, positive nitrate reduction test but shows low catalase activity (Table 2.1). This conventional method is a cheap and simple technique but uses quite a long time for diagnosis (Leite *et al*., 1998).

Table 2.1 Biochemical properties of mycobacteria (Ribón, 2012)

Properties	Type of mycobacteria		
	M. tuberculosis	*M. bovis*	Nontuberculous mycobacteria
Niacin test	+	-	-
Nitrate reduction test	+	-	+/-
68 °C catalase test	-	-	+/-

2.1.3.2 Sputum smear microscopy

This technique is the primary examination method for TB detection in lung or pulmonary TB. It is also a cheap and simple method but is specific only in the density tubercles. The sputum smear microscopy uses Ziehl-Neelsen staining (acid-fast staining) technique for counting the amount of tubercles under microscopy (5000-10,000 cells/ml). In Ziehl-Neelsen staining, carbol fuchsin binds to the mycolic acids in the cell wall of *M. tuberculosis* cells and then, red color of carbol fuchsin is decolorized from other cells or tissues. Fluorochrome such as auramine-O and rhodamine might be used for staining instead of carbol fuchsin. The decolorization step can be performed using 3% (v/v) acid alcohol or 20% (v/v) sulfuric acid. Metylene blue is used for restaining the other cells or tissues except *M. tuberculosis* cells (Desikan *et al*., 2013).

2.1.3.3 Cultivation method

This method is a standard method. Media commonly used for *M. tuberculosis* cultivation are Middlebrook 7H9 broth, Middlebrook 7H10 agar, Middlebrook 7H11, agar egg-based media (Löwenstein-Jensen and Ogawa media) and Bactec 12B. Tween 80 is essentially added in the culture broth in order to disperse the cell colonies. BACTEC 12B is semiautomated radiometric medium which is used in BACTEC 460 TB system (Becton Dickinson, New Jersey, USA) (Fig. 2.2). BACTEC 12B is modified from Middlebrook 7H12 broth supplemented with antibiotics, carbon 12 (^{14}C) labeled palmitic acid that determines the sensitivity of culture in the relationship to histopathology. The obtained result by measuring the volumes of oxygen is reported in a computer. This method is specific and has a high accuracy but it takes a long time for cell cultivation (about 4-6 weeks) (Becker, 1961).

Figure 2.2 BACTEC 460 TB system

Available : http://www.medwow.com/med/microbiological-culture-analyzer/becton-dickinson/bactec-460-tb-system/xbactec-460-tb-system.mth14330_200_200.jpg.pagespeed.ic.CiTe828r31.jpg

2.1.3.4 Polymerase chain reaction (PCR) technique

PCR technique is developed for high potential, accuracy and rapid TB diagnosis. This technique can detect genetic material of *M. tuberculosis* in respiratory specimens such as sputum. PCR technique can amplify with a few templates (genomic DNA) and short times but it has a higher cost than a conventional method. In addition, PCR technique has a limited because *M. tuberculosis* cells in some specimens cannot be detected by PCR.

2.1.3.5 Chromatographic method

Mycolic acids are extracted, reacted with saponification and the obtained products are analyzed by high performance liquid chromatography (HPLC) techniques. The various patterns on chromatography are shown depending on the species of mycobacteria. This technique uses less time but the tool is expensive.

2.1.3.6 Tuberculin skin test

A tuberculin skin test is the first method for TB detection in the lungs. Tuberculin has two types; the Koch's old tuberculin (O.T.) and the Mantoux test (purified protein derivatives (PPD)). The PPD is a collection of protein mixtures and other materials such as nucleic acid, polysaccharides and lipids, filtered from killed *M. tuberculosis* cultures. The Mantoux test was discovered by Charles Mantoux in the twentieth century. The skin test uses a basic principal of proteins and immune response system recognization. The PPD is injected under the layer of skin and then the positive result can be determined from a firm red bump. The disadvantage of skin test is a low sensitivity (Mantoux, 1910).

2.1.4 Anti-tuberculosis antibiotics and resistance mechanisms

The TB treatment uses two types of the effective antituberculosis antibiotics composed of first-line and second-line anti-tuberculosis drugs. First-line anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin) are mainly used in TB treatment because first-line anti-tuberculosis drugs have an antibacterial activity, few side effects and a cheap price. Another type of anti-tuberculosis drug, second-line anti-tuberculosis drugs (fluoroquinolones and aminoglycosides) are used for TB patients has been treated with first-line anti-

tuberculosis drugs resistance treatment. The anti-tuberculosis treatment is separated into two phases, consisting of an initial intensive phase (treatment with three or more first-line anti-tuberculosis drugs such as isoniazid, rifampicin, pyrazinamide and ethambutol) and a continuation phase (treatment with isoniazid and rifampicin). The multidrug initial intensive phase takes about 8 weeks whereas a continuation phase spends approximately 4 months (Guessogo *et al*., 2016).

The emergency of drug-resistance TB is alarming and represents a worldwide health care problem. Multidrug-resistance TB or MDR-TB is TB resistant to at least isoniazid and rifampicin. In addition, extensively drugs-resistance TB or XDR-TB is MDR-TB resistant to any fluoroquinolones and at least one of second-line injectable drugs. First-line and second-line anti-tuberculosis drugs have different structures, activities and resistance mechanisms in *M. tuberculosis*. An investigation on the resistance of these drugs can help patients for TB treatment.

2.1.4.1 First-line anti-tuberculosis drugs activities and their resistance mechanisms

First-line anti-tuberculosis drugs contain various kinds of drugs. Their structures and resistance mechanisms are shown below:

1. Isoniazid

Isoniazid (INH) is a potent highly bacteriocidal antibiotic which has been discovered in 1951 (Timmins *et al*., 2006). INH is commonly used for prophylaxis of healthy household contacts of individuals with Mantoux tuberculin skin test (TST) or in conjunction with other medications for the treatment of active TB infection. This drug passes through the placental barrier and enters into the cells by a passive diffusion (Bardou *et al*., 1998). INH has an empirical formula of $C_6H_7N_3O$. It is structurally consisted of pyridine ring and hydrazine group (Fig. 2.3).

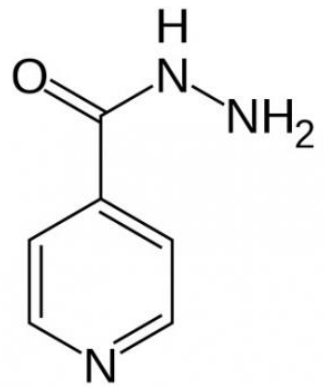


Figure 2.3 Chemical structure of isoniazid (INH)
Available : http://static.framar.bg/thumbs/6/atc/Isoniazid_11.jpg

The control mechanism of INH in bacteria involves many pathways such as mycolic acid synthesis. Normally, INH kills tuberculosis bacilli by inhibition of mycolic acid synthesis (an essential component of *M. tuberculosis* cell wall). In addition, INH acts as an antimetabolite of two major coenzymes, NAD and pyridoxal phosphate. The target of INH in *M. tuberculosis* is InhA protein which is encoded by *inhA* gene. The *inhA* gene encodes an NADH-dependent enoyl-acyl carrier protein reductase which catalyzes the reduction of 2-trans-enoyl chains containing at least 12 carbon atoms (1995, Dessen *et al*., 1995 ; Quémard *et al*.). This enzyme is associated with the step of two-carbon-elongation round in mycolic acid biosynthetic pathway. Therefore, mutation of *inhA* is related to the INH resistance in *M. tuberculosis* (Heym *et al*., 1993 ; Rozwarski *et al*., 1998). In addition, the biofunctional hemoprotein "KatG" is a Class I family of catalase-peroxidase enzyme in *M. tuberculosis* (Table 2.2) (Sherman *et al*., 1999). This enzyme catalyzes the oxidation of various substrates via upwards of two consecutive one-electron oxidation steps utilizing hydrogen peroxide (Fig. 2.4), resulting in INH activity activation. After INH activation, INH-NADH bound with a covalent bond is produced due to the couple reaction of an isonicotinoyl radical and NAD^+/NADH (Fig. 2.5). The resistance of INH in *M. tuberculosis* is a complex process associated with mainly several mutations in *inhA* and *katG*. Mutations of *inhA* promoter region have been found at the position nucleotide -8 to -24 (alternation of cytosine to thymine), resulting in the reduction of NADH binding to Inh*A* or overexpression of *inhA.* The mutation of *katG* at codon 315 (S315T) has been shown as a common point

mutation in clinical isolates that confers a high level resistance to INH (Rawat *et al*., 2003 ; Zhang *et al*., 1992). In addition, other mutations in *ahpC*, *kasA* and *ndh* have been found in INH-resistant *M. tuberculosis*, suggesting that these genes are associated with INH resistance (although the mechanism of resistance is still unknown) (Hazbon *et al*., 2006).

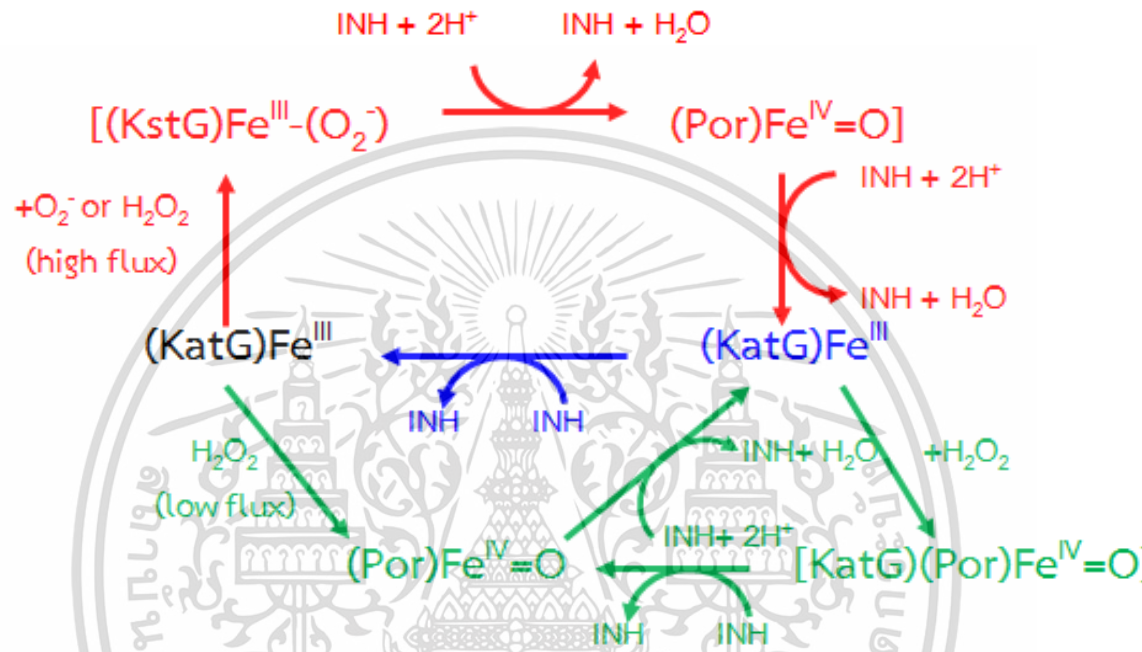


Figure 2.4 Proposed reactions and putative intermadiates of KatG involved in the oxidation of isoniazid (INH)

Available : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2866272/figure/fig02/

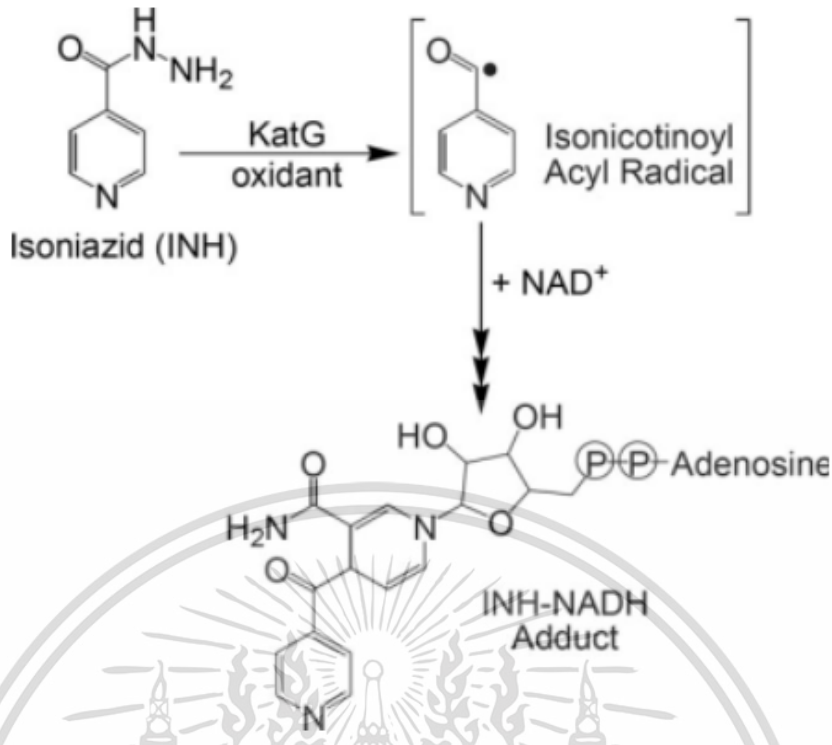


Figure 2.5 Schematic representation of INH-NADH adduct formation as catalyzed by KatG via a putative isonicotinoyl radical

Available : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2866272/figure/fig01/

2. Rifampicin

Rifampicin (RIF) is a semisynthetic derivative rifamycin produced by *Nocardia mediterranei*. RIF is a broad spectrum antibiotic against bacterial infectious photogens and is one of first-line anti-tuberculosis drugs. The discovery of RIF has been started in 1957 and RIF is later introduced as an anti-TB drug in 1972 (Sensi *et al*., 1960 ; Sensi, 1983). RIF and INH are combination drugs for short course treatment of TB. RIF diffuses freely into the living and bacterial cells and makes an extremely effective antibacterial activity against pathogens including *M. tuberculosis* (Shinnick, 1996). The chemically structure of RIF is $C_{43}H_{58}N_4O_{12}$. RIF belongs to a class of macrolide antibiotics that has a propionate-derived chain bridging a tricyclic naphthalene core (Fig. 2.6). The target of RIF is β-subunit of DNA-dependent RNA polymerase. RIF binds with the target, resulting in the blocking of elongation of RNA synthesis. The β-subunit of DNA-dependent RNA polymerase in *M. tuberculosis* is encoded by *rpoB*. This enzyme functions to synthesize the proteins in

M. tuberculosis (Campbell *et al*., 2001 ; Hartmann *et al*, 1967). The mutations in *rpoB* gene have been shown to confer RIF resistance in clinical isolates of *M. tuberculosis* (Table 2.2). The majority of *rpoB* mutation is occurred within 81 bp nt regions or at codon 507-533 which is called “hot-spot region” or “RIF resistance determining region (RRDR)” (Campbell *et al*., 2001 ; Hartmann *et al*., 1967 ; Mboowa *et al*., 2014 ; Ramaswamy *et al*., 1998 ; Telenti *et al*., 1993). Mutation at codon 531 of *rpoB* is the most commonly found in clinical isolates followed by mutation at codon 526 and codon 516 (Casali *et al*., 2012 ; Comas *et al*., 2012 ; Prammanannan *et al*., 2008). The high-level resistance of RIF has been conferred in *M. tuberculosis* clinical isolates with mutations at codon 531 and 526 whereas other mutations in several codons of RRDR region (codon 511, 518, 519, 522, 529 and 533) conferred the low-level resistance to RIF (Comas *et al*., 2012, ; Gagneux *et al*., 2006 ; Prammanannan *et al*., 2008).

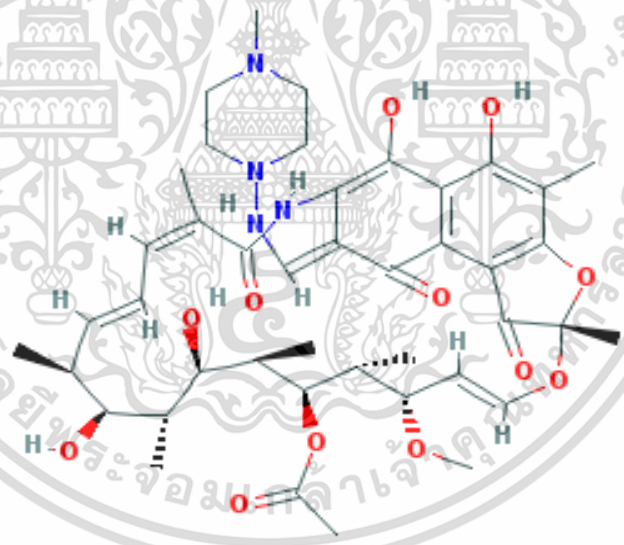


Figure 2.6 Chemical structure of rifampicin (RIF)

Available : https://www.drugbank.ca/structures/DB01045/image.svg

3. Pyrazinamide

Pyrazinamide (PZA) was synthesized for the first time in 1936 and was later recognized as an antituberculous drug in 1952 (Yeager *et al*., 1952). PZA is one of effective drugs for TB treatment. PZA is a nicotinamide analog (Fig. 2.7). The form of PZA is different from other antibiotics because it actions against the growing

bacteria and non-growing persisters. PZA is a prodrug which is changed to pyrazinoic acid (active form) by pyrazinamidase activity under an acidic environment (pH 5.5). This enzyme is encoded by *pncA* in *M. tuberculosis*. After conversion of active form, pyrazinoic acid passes cell membrane and accumulates inside the cells, resulting in the disruption of membrane potential in *M. tuberculosis* (Zhang *et al*., 2009). Thus, mutations in structural gene and promoter region of *pncA* cause the loss of pyrazinamidase activity and finally PZA resistance in *M. tuberculosis* clinical isolates (Table 2.2). The mostly found *pncA* mutation is a deletion at nucleotide 71 that causes a framshift mutation (Scorpio *et al*., 1996 ; Shi *et al*., 2011 ; Yeager *et al*., 1952). The other mutations are a base substitution at nucleotide 11 and 403 (Jonmalung *et al*., 2010 ; Sreevatsan *et al*., 1997 ; Zhang *et al*., 2003).

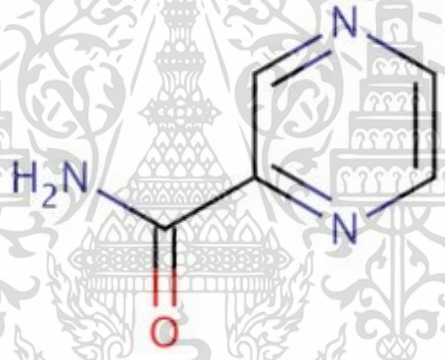


Figure 2.7 Chemical structure of pyrazinamide (PZA)
Available : https://www.drugbank.ca/structures/DB00339/image.png

4. Ethambutol

Ethambutol (EMB) or dextro-2,2′-(ethylenediimino)di-1-butanol has a bacteriostatic activity and was introduced as an anti-TB drug for the first time in 1966 (Karlson *et al*., 1948). EMB is always used in combination with other first-line antituberculous drugs such as isoniazid and rifampicin (Thee *et al*., 2007). The molecular formula of EMB is $C_{10}H_{24}N_2O_2$ (Fig. 2.8). EMB interferes cell wall synthesis with an inhibition of polymerization of arabinan and arabinogalactan (components of cell wall in *M. tuberculosis*). In process of cell wall synthesis, arabinosyl transferase is related to arabinogalactan synthesis and this enzyme is encoded by the 10 kb

embCAB operon (Telenti *et al*., 1997). Furthermore, mutations in *embCAB* confer EMB resistance in *M. tuberculosis* clinical isolates, especially mutations in *embB* (Tabel 2.2) (Johnson *et al*., 2006 ; Plinke *et al*., 2006 ; Takayama *et al*., 1989 ; Telenti *et al*., 1997). The most frequently mutation (about 47-62% of EMB resistant *M. tuberculosis* isolates) has been reported at the codon 306 to confer high-level resistance to EMB in *M. tuberculosis* clinical isolates (Hazbón *et al*., 2005). In addition, other mutations in *embB* at codon 330, 345 and 406 have been also found to confer significantly a level of EMB resistance (Johnson *et al*., 2006 ; Safi *et al*., 2013 ; Telenti *et al*., 1997).

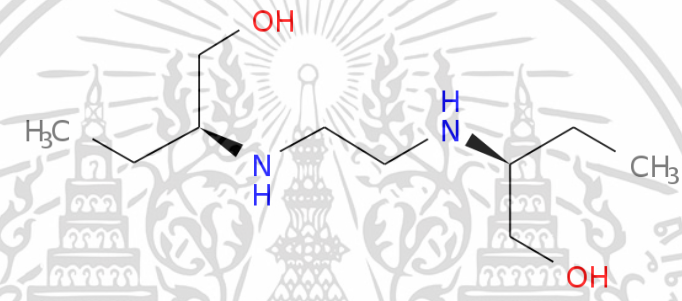


Figure 2.8 Chemical structure of ethambutol (EMB)
Available : https://www.drugs.com/img/mol/DB00330.mol.png

5. Streptomycin

Streptomycin (STR) was discovered as a compound against the tubercle bacillus in 1944 (Julius *et al*., 1978). STR is an aminocyclitol glycoside antibiotic and is used as a combination drug with other first-line antituberculous drugs. The chemical structure of STR is $C_{21}H_{39}N_7O_{12}$ that is consisted of three components of streptidine (inositol with two guanido groups), streptose (methyl pentose) and streptoscamine (N-metyl-L-glycosamine) (Fig. 2.9). STR has common side effects such as vomiting, fever and rush. STR interferes the recognition site of codon-anticodon interaction, resulting in misreading of messenger RNA (mRNA). STR inhibits the initial translation process which affects an inhibition of protein synthesis. Previous reports have demonstrated that STR inhibits peptide synthesis by binding the 30S ribosomal subunit of bacterial ribosome (Finken *et al*., 1993). The STR

resistance in *M. tuberculosis* clinical isolates is caused by mutations in 16S rRNA (*rrs*) and ribosomal protein 12 (*rpsL*) (Zhang *et al*., 2009). The most frequently mutation found in STR-resistant *M. tuberculosis* clinical isolates is mutation in *rpsL* at position K43R (Table 2.2) (Finken *et al*., 1993 ; Nair *et al*., 1993 ; Siddiqi *et al*., 2002 ; Villellas *et al*., 2013). Recently, mutation in *gidB* conferring the low-level STR resistance has been reported in STR-resistant *M. tuberculosis* isolates because *gidB* encodes 16S rRNA methyltransferase (Wong *et al*., 2011).

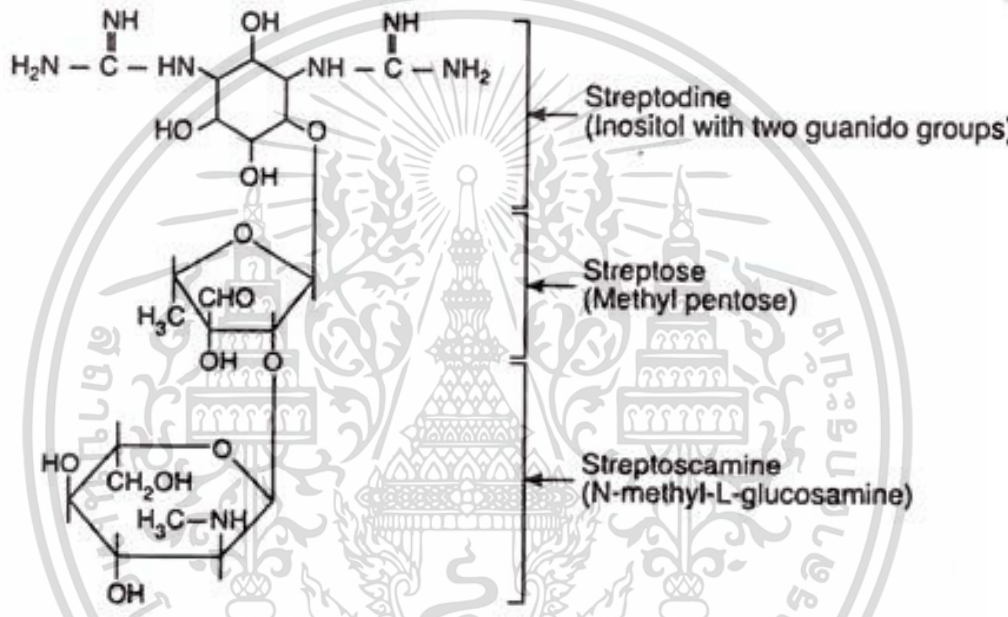


Figure 2.9 Components of streptomycin structure
Available : http://cdn.biologydiscussion.com/wp-content/uploads/2016/09/clip_image002-134.jpg

2.1.4.2 Second-line anti-tuberculosis drugs activities and resistance mechanisms

Fluoroquinolones (FQs) are classified as the second-line antituberculous drugs. They are consisted of lomefloxacin, sparfloxacin, fleroxacin, clincafloxcin, ciprofloxacin, norfloxacin, tosufloxacin, levofloxacin, gatifloxcin and moxifloxacin (Fig. 2.10). FQs are synthetic broad spectrum antibiotics used for a treatment of respiratory disease. Their structures of FQs comprise fluorine,

quinolone, nalidixic and oxolinic acids (Wolfson, 1985). The target of FQs action is DNA gyrase, a type II topoisomerase containing two A and two B subunits, which causes negative supercoiling of the DNA (Takiff *et al*., 1994). The FQs action leads to the inhibition of DNA replication (Hooper, 1999). DNA gyrase was discovered in 1976 in *E. coli* (Gellert *et al*., 1976). In *M. tuberculosis*, the A and B subunits of DNA gyrase are encoded by *gyrA* and *gyrB*, respectively. The FQs resistance mechanisms have been associated with mutations at "Quinolone resistance determining region (QRDR)" of *gyrAB* (Table 2.2). Mutations have also been found at codon 94, 91, 88 and 74 in *gyrA* whereas mutation at codon 95 in *gyrA* is characterized to be a common polymorphism in *M. tuberculosis* clinical isolates. In addition, a mutation at codon 533 of *gyrB* confers gatifloxacin and ofloxacin resistance (Cui *et al*., 2011; Takiff *et al*., 1994; Von Groll *et al*., 2009).

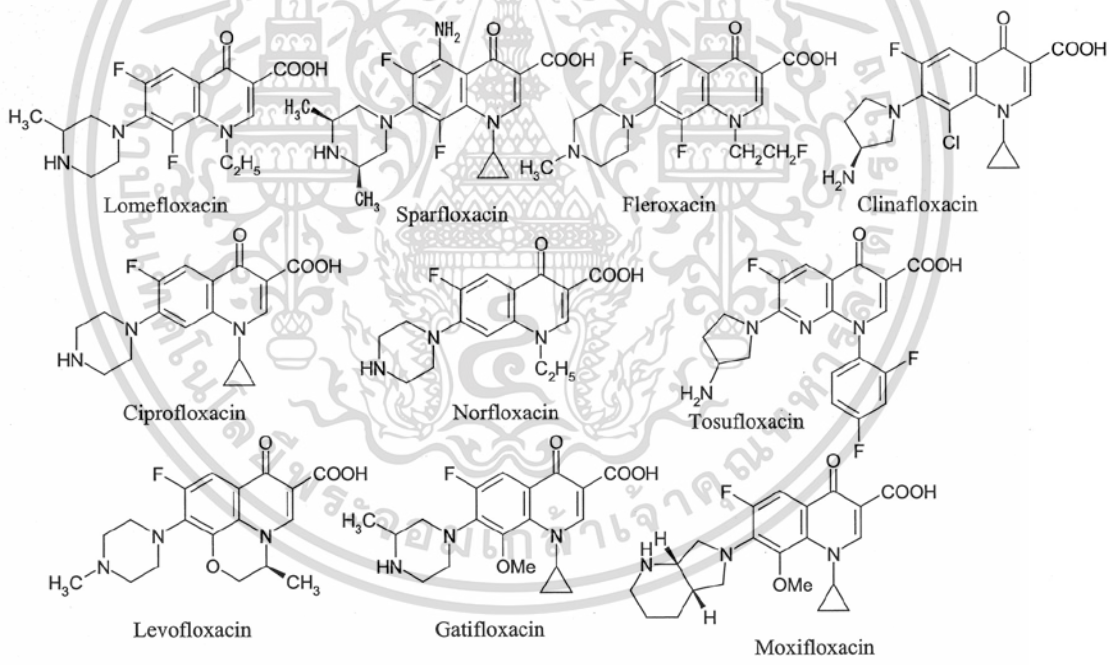


Figure 2.10 Chemical structures of many fluoroquinolones (FQs)
Available: http://aac.asm.org/content/48/3/799/F1.large.jpg

Table 2.2 First-line and second-line antituberculosis drugs profile in *M. tuberculosis*

Antituberculosis drugs	Actions	Drug-resistance genes	References
First-line drugs :			
Isoniazid (INH)	Conversion of an inactive form of catalase-peroxidase (KatG) enzyme by acetylation	*inhA*, *katG* and *ahpC*	Heym *et al*., 1993; Rozwarski *et al*., 1998; Sherman *et al*., 1999
Rifampicin (RIF)	Binding inhibition of ß-subunit of DNA-dependent RNA polymerase	*rpoB*	Campbell *et al*., 2001; Hartmann *et al*, 1967; Mboowa *et al*., 2014
Pyrazinamide (PZA)	Conversion of an inactive form of pyrozionic by pyrazinamidase (PZase)	*pncA*	Yeager *et al*., 1952; Shi *et al*., 2011
Ethambutol (EMB)	Inhibition of arabinosyl transferase (EmbB) activity	*emb*	Takayama *et al*., 1989; Plinke *et al*., 2006
Streptomycin (STR)	Inhibition of peptide synthesis by binding the 30S ribosomal subunit of ribosome	*rrs* and *rpsL*	Finken *et al*., 1993; Siddiqi *et al*., 2002
Second-line drugs : Fluoroquinolones	Binding to DNA gyrase, a type II topoisomerase	*gyrA* and *gyrB*	Takiff *et al*., 1994

2.2 Mycobacteria

2.2.1 Microbiology characteriatics of mycobacteria

Mycobacterium is a bacterium classified in kingdom Bacteria, phylum Actinobacteria, order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*. Nowadays, 60 species of mycobacteria (human pathogen and non-pathogen species) have been identified and named depending on the properties of genetic (16S ribosomal RNA), growth and adansonian taxonomy. Mycobacteria have slender curved rods that are resistant to extreme and toxic environments such as dehydrated, alkaline and acid conditions. Mycobacteria are also identified as acid-fast bacteria because they show an ability of acid resistance by cell wall's structural characterization. Their cell wall contains higher content of waxes and glycolipids than other bacteria.

Types of mycobacteria are categorized upon epidemiology, growth rate, catalase activity, niacin production and pigmentation in light or dark condition. Mycobacteria are separated into two types according to the epidemiology; (1) nontuberculous mycobacteria (NTM) or atypical mycobacteria and (2) *Mycobacterium tuberculosis* complex (MTC). NTM is also called "mycobacteria other than tubercle baciili (MOTT) or mycobacteria of group I-IV". Most bacteria in NTM types are found from several environments (soil, water and dust). They show a lower virulence than other types and some strains can grow in high or low temperature. In addition, NTM are classified into four Runyon's groups by physical properties including growth rate, the biochemical properties and color of colony. Runyon's groups of NTM are presented in Roman number as follows:

Group I – Photochromogen: It is a slow growing mycobacterium that can be seen by naked eyes within 2 weeks of cultivation. The bacteria in this group appear no color in the dark but they produce a yellow-orange pigment during a light cultivation. Yellow color of colony is come from the pigment carotenoid. In this group there are four species caused a disease in human; *Mycobacterium kansasii*, *Mycobacterium simiae*, *Mycobacterium asiaticum* and *Mycobacterium marinum*. *Mycobacterium marinum* causes a skin disease called "Swimming pool granuloma".

Group II – Scotochromogen: It is a slow growing bacterium that produces yellow pigments in the dark cultivation. Most of bacteria in this group do not cause the disease in human but they have been found in human throat, water source and soil. Examples of scotochromogen bacteria are *Mycobacterium szulgai* and *Mycobacterium scrofulaceum.*

Group III – Non photochromogen: It is a slow growing bacterium that does not produce any pigments in the light and dark cultivation. Some non photochromogen bacteria cause diseases in human. They are *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium xenopi* and *Mycobacterium ulcerans*. *M. avium* and *M. intracellulare* are cause of the pulmonary TB like *M. tuberculosis*.

Group IV – Rapid growers: It is a rapid growing bacterium. At the same way, the baciili grow within 2-5 days. Some rapid grower bacteria such as *Mycobacterium fortuitum* have pigments whereas some types of bacilli such as *Mycobacterium phlei* have pigment in the dark.

In 1996, Grange divided the Runyon groups according to the growth rate into the slow growing, rapid growing and non-cultivable mycobacteria (Table 2.3) (Grange, 1996).

The nontuberculous mycobacteria (NTM) have been found in several clinical specimens, for example, sputum and skin. These bacilli affect the inflammation of lungs, nodes, soft tissue, bone and urinary system. The pathogenesis of NTM is similar to MTB by the production of granuloma and caseation. Thus, NTMs caused pulmonary disease like *M. tuberculosis* complex are *M. fortuitum*, *M. avium* complex, *M. kansasii*, *M. xenopai* and *M. malmoense*.

Mycobacterium tuberculosis complex (MTB) includes *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium africanum*. In addition, strains of *M. tuberculosis* are separated into two variants “Classical variant and Asian variant” according to their susceptibility to thiophen-2-carboxylic acid hydrazide (TCH). The classical variant resists to TCH more than Asian variant. At the same way, two variants of *M. africanum* (africanum I and II) and *M. bovis* have been classified by biochemical property (nitrate reduction).

Table 2.3 Runyon groups of mycobacteria (Grange, 1996)

Runyon groups	Groups	Mycobacteria
Slow growing	Photochromogens	*M. kansasii, M. marinum, M. simiae* and *M. asiaticum*
	Scotochromogens	*M. gordonae, M. scrofulaceum* and *M. szulgai*
	Non-chromogens	*M. avium* complex, *M. celatum, M. farcinogenes, M. gastri, M. haemophilum, M. lepraemurium, M. malmoense, M. paratuberculosis, M. shimoidei, M. ulcerans, M. xenopi* and *M. terrae* complex
Rapid growing	Potential pathogens	*M. chelonae* and *M. fortuitum*
	Thermophiles	*M. phlei, M. smegmatis* and *M. thermoresistibile*
	Others	*M. aurum, M. duvalii, M. flavescens, M. gadium, M. gilvum, M. neoaurum, M. chitae, M. parafortuitum, M. senegalense* and *M. vaccae*
Non cultivable		*M. leprae, M. genavense, M. confluentis, M. intermedium* and *M. interjectum*

2.2.2 *Mycobacterium tuberculosis*

M. tuberculosis has been discovered from TB patients by Robert Koch in 1882 (Sakula, 1982). *M. tuberculosis* is an obligated aerobic bacterium that has a rod-shape with 2-4 μm in length and 0.2-0.5 μm in width (Fig. 2.11). The bacilli do not produce capsule or endospore and enable to stain with carbolfuchsin (arylmethane dye) by Ziehl-Neelsen acid-fast staining (Fig. 2.12). *M. tuberculosis* is considered as an acid-fast bacterium because of the characteristic of cell wall compositions.

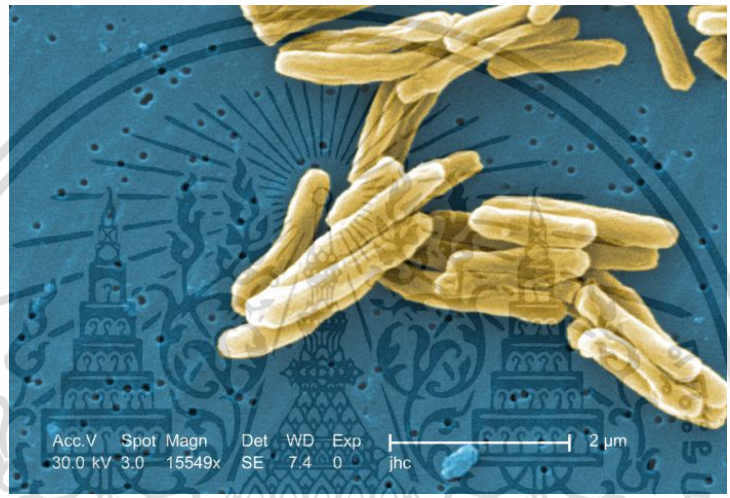


Figure 2.11 *M. tuberculosis* scanning electron micrograph
Available : http://textbookofbacteriology.net/MTBCDC.jpg

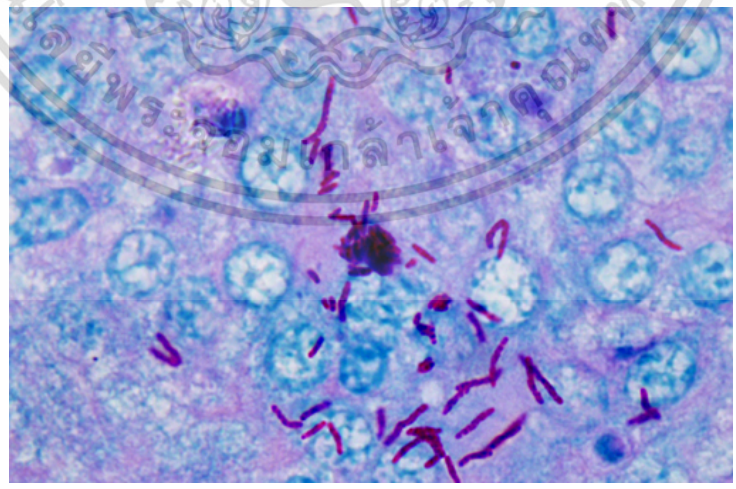


Figure 2.12 Acid-fast staining of *M. tuberculosis* in lung
Available : https://www.askjpc.org/wsco/wsc/images/2012/121102-3.jpg

The cell wall of mycobacterium can protect the toxic substances such as cation proteins, lysozyme and oxygen radicals in the phagocytic granule. The structure of cell wall has three important components, peptidoglycans, arabino galactans and mycolic acids. The combination of three components is called “Mycolyl-arabinogalactan-peptidoglycan complex”. Peptidoglycan layer closes to plasma membrane and arabinogalactans whereas mycolic acid layer is next to arabinogalactans (Fig. 2.13). In addition, cell wall components are covered by mycoside, sulfatide and many lipids. Lipoarabinomannan plays a role as an adherent with phospholipids of plasma membrane (like a skeleton). The arrangement of cell wall peptidoglycan layer in mycobacterium is similar to that in Gram-positive bacteria but cell wall components in mycobacterium are similar to those in Gram-negative bacteria. Cell wall of *M. tuberculosis* has rich lipids that causes the diffusion difficulty of polar compounds and the resistance to acids.

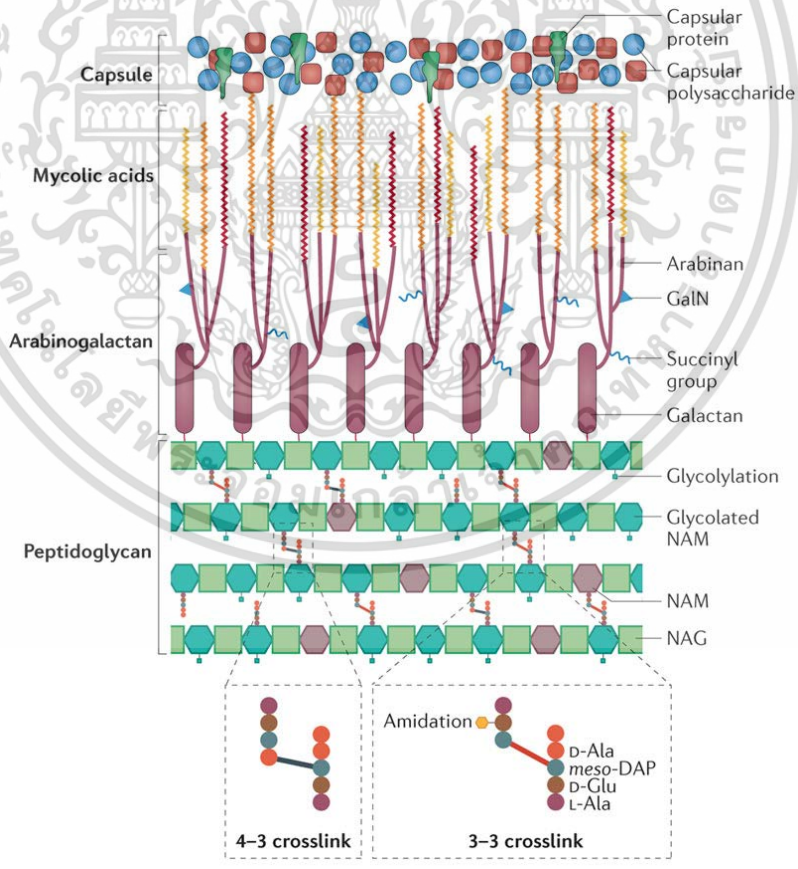


Figure 2.13 Cell wall structure of *M. tuberculosis*

Available : http://www.nature.com/nrmicro/journal/v12/n8/images/nrmicro3299-i1.jpg

The other function of cell wall in *M. tuberculosis* is an antigen. Arabinomannan and arabinogalactan are polysaccharide that causes neutrophilic response and immediate type hypersensitivity in the host. Wax-D, trehalose dimycolate (cord factor) and mycoside C are glycolipids in cell wall and they play a role as important antigen in *M. tuberculosis* and other mycobacteria. Wax-D is peptidoglycolipid "Freund's adjuvant". It enhances immunogenicity of many antigens and affects delayed hypersensitivity. Mycoside C is used to separate serotype in *M. avium* complex and phospholipid enables to activate the humoral antibody in human.

M. tuberculosis grows in the simple media supplemented with carbon (glycerol, pyruvate and glucose) and nitrogen sources (ammonium salt, glutamine and glutamate). In addition, alanine in media and the optimal temperature/pH (38 °C and pH 6.8) help the rapid growth for these bacilli. *M. tuberculosis* likes to grow in rich lipids medium such as Löwenstein Jensen and Ogawa, American Trudeau Society media (ATS) and Petragnani (Elbir *et al*., 2008; Woodruff *et al*., 1946). In medium for *M. tuberculosis* cultivation, the high concentration of oleic acid and tween 80 inhibit bacilli growth whereas their low concentration help the higher growth. Serum albumin is a component in media, for example Middlebrook 7H10 (media supplemented with serum albumin), can absorb fatty acids, making it suitable for *M. tuberculosis* growth. The appropriate concentration of CO_2 (5-10 % (v/v)) causes a high growth of *M. tuberculosis*. The duplication time of *M. tuberculosis* is 12 hours whereas other bacteria use 20 minutes for cell division. In addition, medium containing 5% glycerol helps to separate *M. tuberculosis* and *M. bovis.* The colony form of *M. tuberculosis* is clearly visible to the naked eye within 2 weeks after incubation at 38 °C. The characterization of *M. tuberculosis* colonies on LJ medium is buff color, dry, rough and like a cumulus (Fig. 2.14).

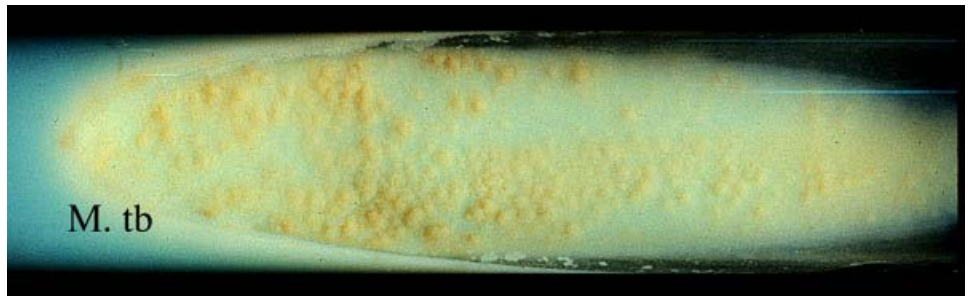


Figure 2.14 Colonies of *M. tuberculosis* on LJ medium
Available : http://www.uaz.edu.mx/histo/pathology/ed/ch_9b/c9b_mtb_mac.jpg

M. tuberculosis can resist to the sunlight, ultraviolet radicals, heat and many agents (3% (v/v) HCl, 2% (w/v) NaOH and 5% (w/v) oxalic acid). The bacilli in sputum can alive for 6 months in dry weather and they can be destroyed by many methods such as heating at 60 °C for 20 minutes, mixing with 70-100% (v/v) ethylalcohol for 10 minutes, mixing with 1% (v/v) formaldehyde for 2 minutes, mixing with 2% (v/v) Lysol for 2 hours and mixing with 5% (v/v) phenol for 1 day.

Mycobacterium has many virulence factors for living in host macrophage. Virulence factors of mycobacterium can be divided into 3 groups based on function, molecular characterization and cellular localization. They are (1) mycolic acids (MAs), (2) trehalose 6,6´-dimycolate (TDM) or cord factor and (3) wax D. Mycolic acids are long chain α-alkyl-β-hydroxy fatty acids that show a hydrophobic property and locate in mycobacterium's cell wall surface (Forrellad *et al*., 2013). The fatty acid contributes the virulence and persistence within a host. Mycolic acid layer covalently links to peptidoglycan, arabinogalactan and other substrates that is a virulence factor TDM. In the other predictions, they probably function in preventing the attack of environmental substrates in phagocytic granule. Cord factor is a glycolipid molecule in cell wall of virulent *M. tuberculosis*. It causes serpentine arrangement of growth and changes a non-toxic to highly toxic within cells. In the same way, wax D is a complex chloroform soluble molecule containing peptidoglycan fragment-arabionogalactan-mycolic acid. It is an effective component for production of certain immunoglobulins in immune system.

2.3 Aminoglycosides

Aminoglycosides (AGs) are effective bactericidal antibiotics against aerobic Gram-negative bacteria. Aminoglycosides contain several drug members, for example, amikacin, kanamycin, streptomycin, gentamicin, neomycin, tobramycin and netilmicin. The first discovered AGs is streptomycin which was isolated from *Streptomyces griseus* (Kroppenstedt *et al*., 2004). The name ending "-micin" is a specific implement for aminoglycoside drug name. AGs are used in the treatment for many infectious diseases including respiratory, abdomen and urological diseases. In the other hand, all AGs cause toxic side effects on kidneys and inner ears. In addition, some AGs such as gentamicin and tobramycin are predominantly vestibulotoxic whereas kanamycin and amikacin are mainly cochleotoxic. Symptom of vestibutoxicity is disequilibrium and dizziness while that of cochleotoxicity is hearing loss and tinnitus.

The basic chemical structure of aminoglycosides is consisted of amino sugars (centre of structure) joined with glycosidic linkages to a dibasic cyclitol or aminocyclitol (Fig. 2.15). Amino sugars are sugars whose hydroxyl groups are replaced with amino groups. An ammonium group of AGs makes a positive charge and polar. Because of high polar property of AGs molecules, they can transport across the outer membrane of bacteria via porin channels. The transport of AGs depends on energy-dependent whereas the block of AGs transport occurs at the low pH and anaerobiosis condition. The aminocyclitol is streptidine which composed of 2-deoxystreptamine and spectinamine. The most clinical AGs antibiotics contain 2-deoxystreptamine in drug structure as found in streptomycin, kanamycin, amikacin, tobramycin and gentamicin.

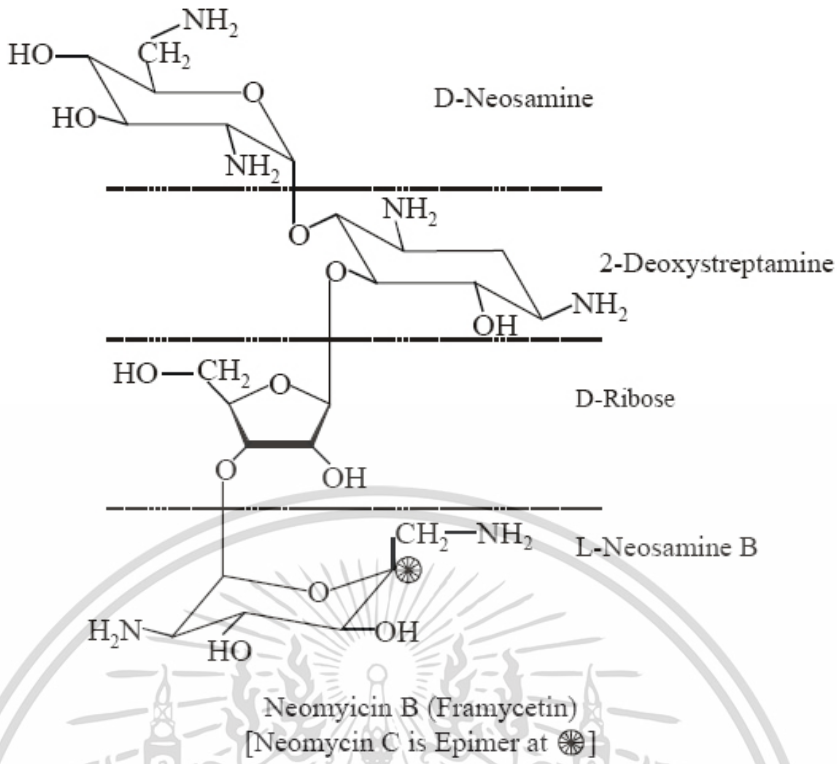


Figure 2.15 Structure of aminoglycosides
Available : http://4.bp.blogspot.com/-iPcDg7yVP8o/UGpXK9AleII/AAAAAAAAFkk/h_BEg72JDlE/s524/08-Neomyicin-B-%2528Framycetin%2529.jpg

AGs bind to 16S rRNA of 30S rRNA ribosomal bacterial subunit at A site in the cytosol, resulting in the inhibition of bacterial protein synthesis. The A site of small ribosomal subunit functions in the movement of aminoacyl tRNA to the next mRNA codon in translation process. Therefore, the interaction at AGs binding site inhibits the elongation chain by increasing of misreading protein or premature termination in translation process that leads to the death of bacterial cells (Fig. 2.16). In addition, some AGs such as amikacin and kanamycin are used as second-line injectable anti-tuberculosis drugs for a clinical treatment.

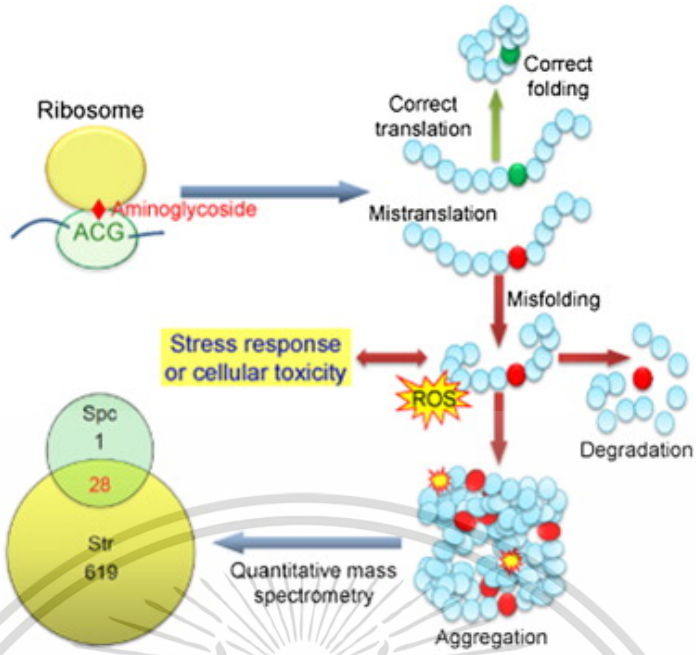


Figure 2.16 Mechanism of aminoglycoside action in bacterial cells
Available : http://www.cell.com/cms/attachment/2016508716/2037209025/fx1.jpg

Kanamycin (KM ; 4,6-diamino-2-hydroxy-1,3-cyclohexane 3,6′diamino-3,6′-dideoxydi-α-D-glucoside) was discovered by Hamao Umezawa in 1957 by isolation from *Streptomyces* species (Umezawa, 1958). KM can action against Staphylococci and Gram-negative organisms, for exemple, *Escherichia coli* and *Salmonella enterica*. KM is structurally composed of amino sugars connected directly with aminocyclitol. The aminocyclitol of KM or 2-deoxystreptamine links to amino sugar at C-atom positions 4 and 6 (Fig 2.17). In addition, KM can be used for tuberculosis treatment. Because of efficiency of KM, it should be used to treat MDR-TB. The side effects of KM are nephorotoxicity, toxicity in otic, irritation and eosinophilia.

Amikacin (AMK; 1-N-(L(-)-gamma-amino-alpha-hydroxybutyryl kanamycin) was introduced in 1976 (Klastersky *et al*., 1976). AMK is a semisynthetic antibiotic that is used for treatment of Gram-negative and MDR-TB infection (Tamma *et al*., 2012). AMK functions to block protein synthesis like other aminoglycoside drugs. AMK is structurally composed of 4,6-disubstituted 2-deoxystreptamines and two sugars linked with glycosidic bond to the central aminocyclitol ring at C-atom positions 4 and 6 (Fig. 2.17) (Bau *et al*., 1999).

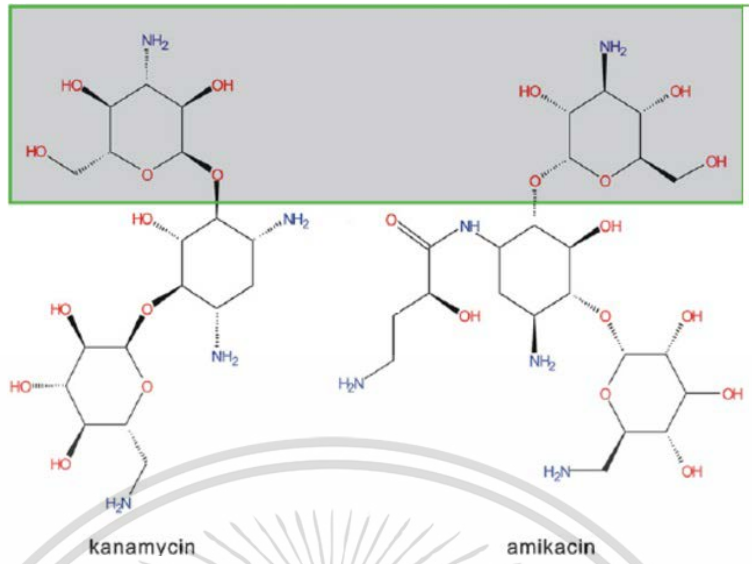


Figure 2.17 Amikacin and kanamycin structure
Available : https://www.researchgate.net/profile/Joel_Pedersen/publication/26267802/figure/fig3/AS:310096199471106@1450944103273/Fig-3-Chemical-structure-of-kanamycin-tobramycin-and-amikacin-Grey-region-shows-the.png

2.4 Aminoglycoside resistance mechanisms

Three AGs-resistance mechanisms have been reported in *M. tuberculosis*. They are (1) an alteration of the ribosomal binding sites, (2) a modification of AGs by enzymatic action and (3) a decrease in drug uptake and accumulation by AGs efflux pump (Fig. 2.18) (Mingeot-Leclercq *et al*., 1999). Understanding of AGs resistance mechanism by studying mutations of genes associated with AGs resistance, genes encoding AGs-modifying enzymes and efflux pump is very important for an effective tuberculosis treatment.

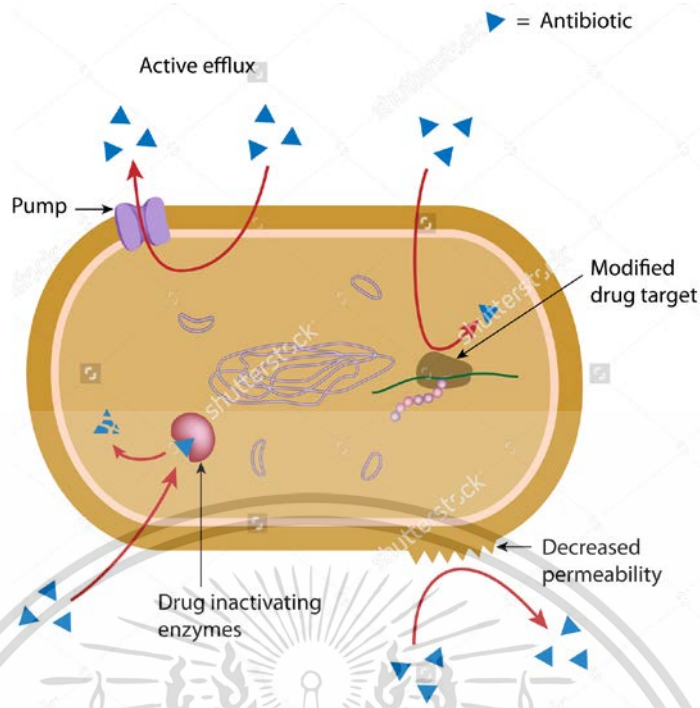


Figure 2.18 Aminoglycoside resistance mechanisms
Available : http://image.shutterstock.com/z/stock-photo-mechanism-of-antibiotic-resistance-in-bacteria-labeled-diagram-166170113.jpg

The aminoglycoside resistance mechanisms are :

(1) An alteration of the ribosomal binding sites

An alteration of the ribosomal binding sites is a main cause of a high-level resistance to AGs in *M. tuberculosis*. This mechanism actions by interfering the binding of AGs with ribosome in the cytosol due to the presence of point mutation. Some reports show the high-level resistance of cross-resistance between KM and AMK. The alteration of the ribosomal binding site causes the high-level resistance to KM and AMK (MICs >64 µg/ml) in MDR-TB and XDR-TB clinical isolates (Jugheli *et al*., 2009 ; Zhang *et al*., 2009 ; Jureen *et al*., 2008). The most frequently mutation in *rrs* at the position A1401G has been found in KM-, AMK-resistant *M. tuberculosis* clinical isolates (Jugheli *et al*., 2009). The *rrs* mutation at position C1402T and G1484T is also found to associate with the cyclic polypeptide capreomycin (CAP), KM and AMK resistance (Table 2.4) (Jugheli *et al*., 2009).

(2) A modification of AGs by enzymatic action

Aminoglycosides (AGs)-modifying enzymes cause a common AGs resistance in mycobacteria. Three enzymes are known to be associated with AGs resistances; nucleotidyltransferases (ANTs), phosphotransferases (APHs) and acetyltransferases (AACs). ANTs catalyze the transfer of adenosine monophosphate (AMP) from substrate adenosine triphosphate (ATP) to hydroxyl group of AGs. There are seven members of ANTs classified in bacteria; ANT(6) (adenylylation at 6-position of AGs), ANT(9) (adenylylation at 9-position of AGs), ANT(4′) (adenylylation at 4′-position of AGs), ANT(2′′) (adenylylation at 2′′-position of AGs), ANT(3′′) (adenylylation at 3′′-position of AGs), ANT(4′)-I and ANT(4′)-II (adenylylation at 4′-position of AGs). ANTs have been identified in *Campylobacter fetus*, *Enterococcus faecalis*, *Bacillus* spp., *Staphylococci* sp. and *P. aeruginosa* (Ramirez *et al*., 2010). APHs catalyze the transfer of a phosphate group to antibiotics. They contain many classes and subclasses such as APH(4)-I, APH(6)-I, APH(2′′) and APH(7′′) (Ramirez *et al*., 2010). Genes coding APHs have been reported in chromosome of *Streptomyces griseus* and *Mycobacterium fortuitum*. The last enzyme AACs contain 160 amino acids and belong to the GNAT-related N-acetyltransferase (GNAT) superfamily of protein (Vetting *et al*., 2005). AACs catalyze an acetylation of NH_2 group at various positions in AGs. AACs have many types such as AAC(1), AAC(3), AAC(2′) and AAC(6′). In *M. tuberculosis*, Eis is an AAC which acetylates multiple NH_2 groups on AGs molecules. The promoter mutations in *eis* have been found in kanamycin-resistant *M. tuberculosis*. These mutations have been correlated to an overexpression of *eis* and a low-level resistance to KM (Zaunbrecher *et al*., 2009).

(3) A decrease in drug uptake and accumulation by AGs efflux pump

This mechanism was found in *Pseudomonas* spp. and other Gram-negative bacilli. Efflux pump benefits to protect cells from the toxic substances or antibiotics. In bacteria, efflux pump superfamilies can be classified either by energy sources or drug-proton antiporters. Six superfamilies of efflux pump have been identified; major facilitator (MFS), ATP-binding cassette (ABC), small multidrug resistance (SMR), resistance-nodulation cell division (RND), multi-antimicrobial extension (MATE) and drug metabolite transporter (DMT) superfamily. In *M. fortuitum*,

Tap is known as a putative efflux pump that has been shown to correlate with tetracycline and aminoglycoside resistance (Aínsa *et al*., 1998).

In another mechanism, WhiB7 is a transcriptional regulator that affects the expression of *eis* and *tap* genes. Point mutations in the 5′-untranslated region (UTR) of *whiB7* (gene encoding WhiB7) have been shown to associate with cross-resistance between second-line anti-tuberculosis drugs due to the upregulation of *eis* and *tap* (Reeves *et al.*, 2013). The point mutations of genes involving in each mechanism of AGs resistance are summarized in Table 2.4.

Table 2.4 Genes associated with aminoglycosides resistance mechanisms in *M. tuberculosis*

Mechanisms of aminoglycosides resistance	Genes (mutation positions)	Drug resistance	References
The alteration of the ribosomal binding sites	*rrs* (A141G, C1402T and G1484T	AMK, KM and CAP	Jugheli *et al*., 2009
Aminoglycoside-modifying enzymes	Promoter of *eis* (C-14T, G-37T, G-10A, C-12T and A-13G)	KM	Zaunbrecher *et al*., 2009
Efflux pump	*tap* or Rv1258c	Tretacyclin and aminoglycoside	Aínsa *et al*., 1998
Others	*whiB7*	Streptomycin	Reeves *et al*., 2013

2.5 Efflux pump

Efflux pump is a common drug resistance mechanism in bacteria. The efflux system has been known for many years. It can extrude toxic substances or antibiotics to the outside of cell membrane (Sarathy *et al*., 2012). The primary function of efflux pump is the transportation of nutrients, molecular signals and metabolic wastes (Aeschlimann *et al*., 2003). Efflux pumps use various energy sources to work such as ATP and substrates to classify into five families. There are major facilitator superfamily (MFS), ATP binding-cassette (ABC) transporter family, multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family and resistance-nodulation-division (RND) superfamily (Putman *et al*., 2000). All superfamilies of efflux pump have been found in Gram-positive bacteria, except RND superfamily. These efflux pump proteins could be found in the inner- and outer-membrane of bacteria. They are encoded by many genes such as *acrB* in *E. coli*, *mexB* in *P. aeruginosa*, *norA* in *Staphylococcus aureus* and *pmrA* in *Streptococcus pneumonia* (Piddock, 2006). In bacteria, AcrAB-TolC efflux pump enables to extrude many antibiotics which are quinolones, chloramphenicol, nalidixic and tetracycline (Poole *et al*., 2002 ; Nishino *et al*., 2009). Genes encoding efflux pumps of only four superfamilies have been reported in *M. tuberculosis* (Table 2.5) (Balganesh *et al*., 2012).

The efflux pump is structurally composed of twelve transmembrane helices with H3, H6, H9 and H12 helices for transportation lipophilic compounds and hydrophobic residues in the internal cavity (Yin *et al*., 2006). The structure of RND efflux pump is a symmetric trimer structure. This trimer contains periplasmic headpiece (docking domain) and transmembrane region (Murakami *et al*., 2002). In addition, efflux pumps have many roles in bacterial cells such as role in virulence, cell-to-cell communication, biofilm formation and drug resistance mechanism.

In *M. tuberculosis*, efflux pump is an important mechanism for drug resistance and it has many functional and unfunctional transmembrane proteins. The study and development the knowledge about efflux pumps mechanism is facilitate to TB treatment.

Table 2.5 Functional and unfunctional transmembrane genes in *M. tuberculosis*

Efflux pump groups	Genes	Substrates	References
MFS	Rv0783c	Rifampicin	Pang *et al*., 2013
	Rv1250	Isoniazid	Li *et al*., 2015
	Rv1258c	Tetracycline, Rifampicin, Aminoglycosides	Aínsa *et al*., 1998
	Rv1410c	Rifampicin, Clofazimine, Tetracycline, Aminoglycosides	Silva *et al*., 2011
	Rv1634	Fluoroquinolone	Li *et al*., 2015
	Rv1877	Ethidium bromide, Acriflavine, Erythromycin	Li *et al*., 2004
	Rv2333c	Spectinomycin, Tetracycline	RamÓn-García *et al*., 2007
	Rv2846c	Isoniazid	Gupta *et al*., 2010
	Rv3239c	Not determined	-
ABC	Rv0194	Ampicillin, Chloramphenicol, Aminoglycosides, Novobiocin	Hao *et al*., 2011
	Rv1456c	Isoniazid	-
	Rv1457c	Rifampicin	-
	Rv1458c	Streptomycin, Ethambutol	-
	Rv1819c	Bleomycin	Domenech *et al*., 2009
RND	Rv1145	Not determined	-
	Rv1146	Not determined	-
SMR	Rv3065	Erythromycin, Thioridazine	Balganesh *et al*., 2012

CHAPTER 3

Research Methodology

3.1 Bacterial strains

3.1.1 Twenty-nine of KM-resistant and 27 of KM-susceptible *Mycobacterium tuberculosis* clinical strains were obtained from the Drug Resistance Tuberculosis Research Fund under the Patronage to Her Royal Highness Princess Galayani Vadhana Krom Luang Naradhiwas Rajanagarindra, Faculty of Medicine, Siriraj hospital. They were isolated from different patients. This study was approved by the Siriraj Ethics Committee, Mahidol University, Bangkok, Thailand (Certificate of Approval No. Si 208/2005).

3.1.2 *Escherichia coli* DH5α (fhuA2 Δ(argF-lacZ)U169 phoA glnV44 *Φ80* Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1hsdR17)

3.1.3 *Mycobacterium tuberculosis* H37Ra ATCC 25177 was obtained from the Tuberculosis Research Laboratory, National Center for Genetic Engineering and Biotechnology.

3.14 *Mycobacterium tuberculosis* H37Rv ATCC 27294 was obtained from the Tuberculosis Research Laboratory, National Center for Genetic Engineering and Biotechnology.

3.2 Plasmids

3.2.1 pDrive TA cloning Vector (Appendix A) (Qiagen, Hilden, Germany)

3.2.2 pSMT1 vector (Snewin *et al*., 1999) was obtained from the Tuberculosis Research Laboratory, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand (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, New Jersey, USA)

3.3.1.3 Middlebrook 7H9 medium (Appendix B) (Becton Dickinson, New Jersey, USA)

3.3.1.4 Super optimized broth (SOB) medium (Appendix B)

3.3.1.5 Oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, New Jersey, USA) (Appendix B)

3.3.1.6 Löwenstein-Jensen (LJ) medium (Becton Dickinson, New Jersey, USA)

3.3.2 Antibiotics

3.3.2.1 Kanamycin (Sigma Aldrich, Steinheim, Germany)

3.3.2.2 Amikacin (Sandoz, Holzkirchen, Germany)

3.3.2.3 Capreomycin (Sigma Aldrich, Steinheim, Germany)

3.3.2.4 Hygromycin (Sigma Aldrich, Steinheim, Germany)

3.3.3 Enzymes

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

3.3.3.2 RNase A (Qiagen, Hilden, Germany)

3.3.3.3 DNase I (Invitrogen, Massachusetts, USA)

3.3.3.4 Lysozyme (Amresco, Ohio, USA)

3.3.3.5 Alkaline phosphatase, Calf intestinal (CIP) (Biolabs, London, England)

3.3.3.6 M-MuLV reverse transcriptase (Thermo Scientific, Massachusetts, USA)

3.3.3.7 Premi Ex *Taq*^{TM} DNA polymerase (Takara, Tokyo, Japan)

3.3.3.8 *Bam*HI (Biolabs, London, England)

3.3.3.9 *Eco*RI (Biolabs, London, England)

3.3.3.10 T4 DNA ligase (Takara, Tokyo, Japan)

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)

3.3.4.3 2-Log DNA ladder DNA (Biolabs, London, England)

3.3.5 Chemicals

3.3.5.1 Agarose (Research Organics, Ohio, USA)

3.3.5.2 TRIzol™ reagent (Thermo Scientific, Massachusetts, USA)

3.3.5.3 Bacteriological agar (Scharlau, Barcelona, Spain)

3.3.5.4 Boric acid (Merck, Darmstadt, Germany)

3.3.5.5 Calcium chloride (Scharlau, Barcelona, Spain)

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

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

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

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

3.3.5.10 Ethanol (Fisher, Springfield, USA)

3.3.5.11 GelStar® (Cambrix Bio Science, Rockland, USA)

3.3.5.12 Glacial acetic acid (Labscan, Dublin, Ireland)

3.3.5.13 Glycerol (Fluka, Buchs, Schweizerland)

3.3.5.14 Hydrochloric acid (Labscan, Dublin, Ireland)

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

3.3.5.16 Manganese chloride (Scharlau, Barcelona, Spain)

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

3.3.5.18 Peptone (Biomark™, Pune, India)

3.3.5.19 Potassium chloride (Scharlau, Barcelona, Spain)

3.3.5.20 Potassium acetate (Fisher, Springfield, USA)

3.3.5.21 Proteinase K (USB Corp, Cleveland, USA)

3.3.5.22 Sodium chloride (Ajax, Finechem, New Zealand)

3.3.5.23 Sodium dodecyl sulfate (Promega, Madison, USA)

3.3.5.24 Sodium hydroxide (Labscan, Dublin, Ireland)

3.3.5.25 Tris-EDTA (TE) (USB Corp, Cleveland, USA)

3.3.5.26 Tween 80 (Fluka, Buchs, Schweizerland)

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

3.3.5.28 Yeast extract (Himedia®, Mumbai, India)

3.3.6 Kits

3.3.6.1 PureLink® RNA Mini Kit (Ambion, Texas, USA)

3.3.6.2 High-Speed Plasmid DNA Mini kit (Geneaid, New Taipei City, Taiwan)

3.3.6.4 Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan)

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 96-Well Microtiter™ Microplates (Thermo Scientific, Massachusetts, 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)

3.4.18 PikoReal™ 24-well Real-Time PCR System (Thermo Scientific, Massachusetts, USA)

3.5 Growth conditions

3.5.1 Mycobacterial growth conditions

M. tuberculosis strains were retrieved from -80 °C stock cultures and subcultured on Löwenstein-Jensen (LJ) medium (Becton Dickinson, New Jersey, USA). The cultures were incubated at 37 °C for 4 weeks. Before performing the experiment, KM-resistant and KM-susceptible strains were subcultured on Middlebrook 7H10 supplemented with 10% OADC and 6 µg/ml of kanamycin and incubated at 37 °C for 4 weeks. KM-resistant and KM-susceptible *M. tuberculosis* H37Rv and H37Ra were grown on Middlebrook 7H10 supplemented with 10% OADC. All strains were incubated at 37 °C for 4 weeks.

3.5.2 *Escherichia coli* growth condition

E. coli was cultured on LB agar (Appendix B) at 37 °C for overnight or in LB broth at 37 °C with shaking speed of 250 rpm for overnight.

3.6 Molecular analysis methods

3.6.1 Nucleic acid isolation

3.6.1.1 *M. tuberculosis* genomic DNA isolation

A loopful of *M. tuberculosis* colonies was scraped from the agar plate and suspended in 500 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH8.0)]. Cells were washed in 500 µl of TE buffer and inactivated by heating at 80 ℃ for 20 min before harvesting cells by centrifugation at 12,000 xg for 5 min. The 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 ℃ for 3 h. SDS and proteinase K were added into the cell suspension at final concentrations of 1% (w/v) and 1 mg/ml, respectively, and incubated at 37 ℃ for 1 h. The reaction of proteinase K was stopped by heating at 65 ℃ for 10 min. Eighty microliters of the mixture of 5 M NaCl and 10% (w/v) CTAB in 0.7 M NaCl was added and the suspension was heated at 65 ℃ for 15 min. The equal volume of chloroform-isoamylalcohol (24:1) (v/v) was added and mixed vigorously. The upper aqueous phase was separated by centrifugation at 12,000 xg for 5 min and transferred into a new microcentrifuge tube. The aqueous phase was re-extracted once with the same volume of chloroform-isoamylalcohol (24:1) (v/v). DNA was precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.3) and 2.5 vol of ice-chilled absolute ethanol, followed by incubation at -70 ℃ for 30 min. Total nucleic acid was isolated by centrifugation at 12,000 xg at 4 ℃ for 15 min, washed with 500 µl of ice-chilled 70% (v/v) ethanol before centrifugation again at 12,000 xg at 4 ℃ for 5 min. The DNA pellet was dried at room temperature and resuspended in 20 µl of TE buffer. RNA was digested by adding 1 µl of 10 mg/ml RNaseA before incubation at 37 ℃ for 1 h. DNA concentration was determined using spectrophotometer by measuring absorbance at wavelength of 260 nm and 280 nm.

3.6.1.2 *M. tuberculosis* total RNA isolation

A loopful of *M. tuberculosis* cells was resuspened in 200 µl of TE buffer pH (8.0). The cell pellet was centrifuged at 12,000 xg at 4 ℃ for 2 min. Total RNA was extracted by adding TRIzol (Thermo Scientific, Massachusetts, USA) and sample was vortexed vigorously for 15 s. The total RNA was precipitated by adding one vol of absolute ethanol, followed by mixing and centrifugation at 12,000 xg at 4 ℃ for 30 s. Total RNA was purified using PureLink® RNA Mini Kit (Ambion, Texas, USA). Briefly, the sample was transferred into a spin cartridge with a collection tube

and centrifuged at 12,000 xg at 4 °C for 15 s at room temperature. The flow-through was discarded and 700 µl of Wash Buffer I into a spin cartridge. The mixture was centrifuged at 12,000 xg at 4 °C for 15 s and the flow-through in collection tube was then discarded. The spin cartridge was placed into a new collection tube and 500 µl Wash buffer II containing ethanol was added to the tube before centrifugation at 12,000 xg at 4 °C for 2 min and flow-through was discarded. This step was repeated and the spin cartridge was centrifuged again at 12,000 xg at 4 °C for 2 min for drying a membrane attached RNA. The spin cartridge was transferred into a recovery tube and RNA was eluted by adding 50 µl of RNase-Free water to the center of the spin cartridge. Finally, the spin cartridge containing RNA was incubated at room temperature for 1 min and centrifuged at 12,000 xg at 4 °C for 2 min. The total RNA was treated by adding 10 µl of *DNase*I reaction buffer and 2.5 µl of *DNase*I (Invitrogen, Massachusetts, USA) and incubated at 37 °C for overnight.

3.6.1.3 Plasmid DNA isolation of *Escherichia coli*

High-Speed Plasmid DNA Mini kit (Geneaid, New Taipei City, Taiwan) was used for plasmid DNA isolation. A single colony of bacteria was picked and suspended into LB broth containing 50 µg/ml of kanamycin. The bacteria were incubated at 37 °C with shaking at 250 rpm for overnight. Cells were harvested by centrifugation at 12,000 xg at room temperature for 1 min and then resuspended in 200 µl of PD1 buffer containing RNaseA. Two hundred microlitres of PD2 buffer were added to the cell suspension and the mixture was gently mixed by tube inversion. In neutralization step, 300 µl of PD3 buffer were added and the mixture was immediately mixed by tube inversion. The PD column and the collection tube were prepared and cell debris was pelleted by centrifugation at 12,000 xg at room temperature for 3 min. The mixture was transferred into the PD column and centrifuged at 12,000 xg at room temperature for 1 min, followed by discarding the liquid in the collection tube. The column was washed by adding 400 µl of W1 buffer and centrifuged at 12,000 xg for 30 s. Six-hundred microlitres of Wash buffer containing ethanol were added to the column and the column was subsequently centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for 3 min. The column was transferred and placed on the new microcentrifuge tube and 50 µl of elution buffer were added into the center of the

column. The column was placed at room temperature for 1 min. Plasmid DNA was eluted by centrifugation at 12,000 xg at room temperature for 3 min and stored at -20 °C.

3.6.2 Determination of nucleic acid concentration

3.6.2.1 Estimation by agarose gel electrophoresis

For standard minigels, 0.8% (w/v) agarose gel was prepared by dissolving 0.16 g of powdered agarose in 20 ml of TBE buffer [89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA (pH8.3)] in Erlenmeyer flask. The slurry was heated in a microwave until the agarose completely dissolves. GelStar® staining solution (Cambrix Bio Science, Rockland, USA) or ethidium bromide was added to the warm agarose gel to make a final concentration of 1X (from 10,000X concentrated Gelstar solution) or 5 µg mL^{-1} ethidium bromide and the gel was mixed by gentle swirling. An appropriate comb was chosen for forming the sample slots in the gel. The warm agarose gel was poured into a casting and allowed to set solid completely (15-20 min at a room temperature). Before electrophoresis, the gel was transferred to the electrophoresis tank and TBE buffer was filled to cover the gel. DNA sample was mixed with tracking dye and loaded into the well. Five-hundred ng of standard DNA fragments (λ DNA/*Hin*dIII fragments, 100-bp ladder DNA and 2-Log DNA ladder DNA) were used as DNA marker. The electric current of 8 Vcm^{-1} was applied to the gel. After electrophoresis, the gel was analyzed under an ultraviolet light.

3.6.2.2 Spectrophotometric quantification

Two µl of nucleic acid sample were diluted in 98 µl TE buffer (pH 8.0) in microcentrifuge tube. The nucleic acid sample was transferred into cuvette and TE buffer was used as a blank. The absorbance of sample was determined by spectrophotometer at wavelengths 260 nm and 280 nm. The quantitative analysis and purification of nucleic acid concentration were performed as described in 1982 (Maniatis *et al*., 1982).

3.6.3 Total nucleic acid purification

3.6.3.1 Purification of PCR products

PCR products were purified using the Gel/PCR DNA Fragments Extraction kit (Geneaid, New Taipei City, Taiwan). Five vol of DF buffer were mixed with PCR products by vortexing. The mixture was transferred to the DF column and centrifuged at 12,000 xg for 30 s. The flow-through was discarded, and the column was washed by adding 600 µl of wash buffer, and centrifuged at 12,000 xg for 30 s. The flow-though was discarded. The column was centrifuged again for 2 min and placed into a new microcentrifuge tube. DNA was eluted from the column by adding 20 µl of elution buffer and centrifuging before centrifugation for 2 min.

3.6.3.2 Purification of DNA fragment from agarose gel

DNA was purified from agarose gel by using the Gel/PCR DNA Fragments Extraction kit (Geneaid, New Taipei City, Taiwan). The agarose gel slice containing relevant DNA fragment was excised and transferred to a microcentrifuge tube. The 500 µl of DF buffer was added into the sample and mixed by vortexing. The mixture was incubated at 60°C for 10 min until the gel slice was completely dissolved. After incubation, the mixture was transferred to DF column and centrifuged at 12,000 xg for 30 s the flow-through was discarded. DNA fragment was washed by adding 400 µl of W1 buffer and the column was centrifuged at 12,000 xg for 30 s. Six-hundred µl of wash buffer containing ethanol were added and the column was stood for 1 min at room temperature. The centrifugation was performed at 12,000 xg at room temperature for 30 s and the flow-through was discarded. The column was centrifuged again at 12,000 xg at room temperature for 2 min and placed into a new microcentrifuge tube. DNA was eluted by adding 30 µl of elution buffer and before incubation for at least 2 min and centrifugation at 12,000 xg at room temperature for 2 min.

3.6.4 Drug susceptibility testing

3.6.4.1 Drug susceptibility testing by proportion method

Drug susceptibility testing (DST) was carried out using the disc elution method on Middlebrook 7H10 agar (Difco, Detroit, Michigan, USA) supplemented with 10% (w/v) oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson,

USA) as recommended by the CLSI (CLSI, 2003). Briefly, the appropriate drug discs were dispensed aseptically into the center of individual quadrants of sterile plastic dishes. Exactly 5.0 ml each of sterile M7H10 medium was pipetted over the discs, and the plates were left overnight at room temperature to permit the drug to diffuse uniformly. The inoculum was prepared by suspension of the *M. tuberculosis* cells in Middlebrook 7H9 broth with the turbidity adjusted to match cell density of McFarland No. 1 standard. The cell suspension was diluted to 10^{-2} (approx. 10^5 CFU/ml) and 10^{-4} (approx. 10^3 CFU/ml) in sterile distilled water; these two dilutions were inoculated onto each quadrant and onto a quadrant containing a drug-free medium, which was used as a control. The plate was incubated at 37 °C until colonies appeared on the control quadrant (approximately 2–4 weeks). Resistance was reported when the colonies on the drug-containing quadrant appeared ≥1% compared to the drug-free control quadrant. For quality control, *M. tuberculosis* H37Rv ATCC 27294 was used as a control for DST. This strain was used at each time when a batch of DST was set up. If any resistance was observed in the control strain, all the results in that batch were not interpreted.

3.6.4.2 Determination of minimum inhibitory concentration (MIC) by agar dilution method

Inoculums were prepared by scraping colonies of exponential growth of *M. tuberculosis* from solid medium. Colonies were dispersed in a screw-cap tube containing Middlebrook 7H9 broth supplemented with 10% (w/v) OADC, 0.05% (v/v) Tween 80 and 5 glass beads (diameter of 6 mm). Mixture was mixed by vortexing at room temperature for 2-3 min and stood at room temperature for 20 min. Cell suspension was transferred to new sterile tube and adjusted to a turbidity equivalent to McFarland No. 1 standard (3 X 10^7 cells/ml) with Middlebrook 7H9 broth. Five µl of cell suspension were spotted on Middlebrook 7H10 agar supplemented with amikacin, kanamycin and capreomycin at final concentration of 0, 2, 4, 8, 16, 32 and 64 µg/ml. All plates were dried and incubated at 37 °C for 3 weeks. Determination of MIC is shown as the lowest antibiotic concentration that found <1% of colonies on colony compared with control plate (Middlebrook 7H10 withot antibiotics) (Sirgel *et al*., 2009).

3.7 Genetic characterization of amikacin (AMK), kanamycin (KM) and capreomycin (CAP) resistance

The nucleotide sequences of genes associated with aminoglycosides (AMK and KM) and polypeptide antibiotic (CAP) resistance, namely *rrs*, *eis* including promoter region, *tap*, *whiB7*, and *tlyA*, were investigated in KM-resistant and –susceptible *M. tuberculosis* clinical isolates. The involving genes using genomic DNAs of *M. tuberculosis* strains as templates were amplified by PCR and submitted to DNA sequencing.

3.7.1 Primer design for PCR amplification

Forward and reverse primers were designed to bind specifically and amplify the products covering about 500 bp upstream and 100 bp downstream of the gene, resulting in approximately 800-1,800 bp of PCR product sizes. Melting temperatures of each primer were shown in Table 3.1. The PCR primer calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe *et al.*, 2017) was used to design all primers based on the sequence of *M. tuberculosis* H37Rv (Accession no. NC_000962). All primers were checked for possible GC content, hairpin formation and primer dimerization using ClustalW programme.

Table 3.1 Primers for amplification and sequencing of genes associated with KM resistance in *M. tuberculosis* H37Rv

KM-resistant genes	Primer	Primer sequence (5′⟶3′)	Tm (°C)	PCR product size (bp)	Reference
PCR primers					
rrs	F-rrs PCR	TTCTAAATACCTTTGGCTCCCT	51	1,680	Daum et al.,
	R-rrs PCR	TGGCCAACTTTGTTGTCATGCA	53		2012
eis (Rv2416c)	F-Rv2417c[a]	GCGGTGCATCACGTCGCCGA	60	1,660	This study
	R-eis-Rv2415c[a]	GCAACGCGATCCGCGAGTGC	60		
tap (Rv1258c)	F-Rv1259[a]	CAGGCCGGCCCTATGCAGTG	58	1,847	This study
	R-Rv1257c[a]	CGGTCTTGCCGGTAGCCGTC	60		
tlyA (Rv1694)	F-tlyA[a]	GTGGCACGACGTGCCCGCGT	60	807	This study
	R-tlyA[a]	CTACGGGCCCTCGCTAATCG	58		
whiB7 (Rv3197A)	F-URT whiB7[a]	GCTGGTTCGCGGTCGGACCT	62	550	This study
	R-whiB7[b]	CGGGGTATCGGCGAACCACA	58		
Sequencing primers					
rrs	F-rrs1	CTGGGCGTAAAGAGCTCGTA	54	-	This study
	F-rrs2	GTTGCCAGCACGTAATGGTG	54	-	This study
	R-rrs1	TCCACCTACCGTCAATCCGA	54	-	This study
	R-rrs2	ATCTCACGACACGAGCTGAC	54	-	This study
eis (Rv2416c)	F-eis1	AGTTTCGTCGCGGTGGCGCC	60	-	This study
	F-eis2	GGACCCGTTACCCCACCTGC	60	-	This study
	R-eis1	GGCGGTCGGGAGCACCACTT	60	-	This study
	R-eis2	TCAGGGCCCGCCACAACGCA	60	-	This study
Tap (Rv1258c)	F-tap1	TCGCAACGCTGATGGCGGCC	60	-	This study
	F-tap2	AGGGGCTGCGCTTCGTCTGG	60	-	This study
	R-tap1	CCCGAAGTAGTCGACCGCGG	60	-	This study
	R-tap2	GACGGGGAACGCGGATAGCC	60	-	This study

[a] Primers used for both PCR amplification and sequencing.

3.7.2 DNA amplification by polymerase chain reaction (PCR)

PCR was performed in a 50 µl reaction mixture shown in Table 3.2. PCR condition consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing and extension (72 °C) step depending on the melting temperatures (Table 3.1) and PCR product sizes (1,000 base pairs per 1 min for extension times). In the final cycle, an additional extension at 72 °C for 7 min was performed. PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis and purified using the Gel/PCR fragment purification Kit (Geneaid, Taiwan) (Section 3.6.1.3) prior for further investigation.

Table 3.2 PCR reaction for amplification of KM-resistant genes in *M. tuberculosis*

Components	Volume (µl)
10X PCR buffer	5
25 mM of $MgCl_2$	3
10 mM of dNTPs	1
5 µM of F primer	2.5
5 µM of R primer	2.5
Taq DNA polymerase (5 U/µl)	0.5
DNA template (50 ng/µl)	1
Deionized water	34.5
Total	**50**

3.7.3 Nucleotide sequencing and analysis

PCR products or recombinant plasmid DNAs were sequenced using the Big-Dye^{TM} terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) and ABI PRISM^{R} 3700 DNA analyzer at First BASE Laboratories (Malaysia). Nucleotide sequences of PCR products were analyzed by comparing them with the sequence of *M. tuberculosis* H37Rv deposited in Genbank database (Accession No. AL123456) by pairwise alignment using the ClustalW program (Thompson *et al*., 1994). Primers for sequencing of each gene were shown in Table 3.1.

3.8 Whole genome sequencing of KM-resistant *M. tuberculosis* MT433 strain

3.8.1 Determination of genome sequence of KM-resistant *M. tuberculosis* MT433 by Next generation sequencing

The KM-resistant *M. tuberculosis* strain without any known mutations was submitted for whole genome sequencing (WGS). The quality and quantity of genomic DNA of *M. tuberculosis* MT433 (DS no. 24433) were determined by 1% (w/v) agarose gel electrophoresis (section 3.6.2.1) and by spectrophotometric method (section 3.6.2.2). One µg of genomic DNA was prepared and submitted for whole genome sequencing on an Illumina HiSeq 2000 platform (generating 100 base pair read lengths) (Illumina, San Diego, CA, USA) at Macrogen Inc., (Seoul, South Korea).

3.8.2 Whole genome sequencing analysis pipeline

The workflow of whole genome sequencing was summarized in Fig. 3.1. Adapter sequence trimmed FastQ files of sample was obtained from Macrogen Inc., (Seoul, South Korea). Quality of reads was checked using a quality control tool for high throughput sequence data (available software FastQC version 0.10.1; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). FastA sequence of the reference *M. tuberculosis* H37Rv (GenBank accession no. NC_000962.3) was retrieved from NCBI GenBank database. The alignment of paired-end raw reads with the reference *M. tuberculosis* H37Rv was performed using Bowtie2 (version 2.1.0) (fast and sensitive read alignment program) and the determination of read coverage over the reference genome was done by bedtools (version 17.2.0) (available: https://github.com/arq5x/bedtools2/releases) (a power toolset for genome arithmetic) (Langmead *et al*., 2009). Aligned reads of sample were sorted, indexed and combined using SAM tools (Li *et al*., 2009). The variant calling was performed using VerScan version 2.2.11 that had default parameters, including in the minimum coverage of 8X, minimum alternative read of 2, minimum average quality of 15 and minimum variant frequency of 0.02 (Koboldt *et al*., 2012). The low quality of small nucleotide variants was cut off using cutoff Phred quality score. In addition, a heterozygous small nucleotide variant with a minimum allele frequency of 75% was discarded (errors). Synonymous, nonsynonymous, insertion and deletion were

classified in the sequence but the variants of abundant antigens Pro-Glu (PE), Pro-Pro-Glu (PPE), polymorphic GC-rich sequence (PE_PGRS) were discarded (Comas *et al*., 2010). Positions of efflux pump genes variants were observed by Intergrative Genomics Viewer (IGV) (Thorvaldsdottir *et al*., 2013).

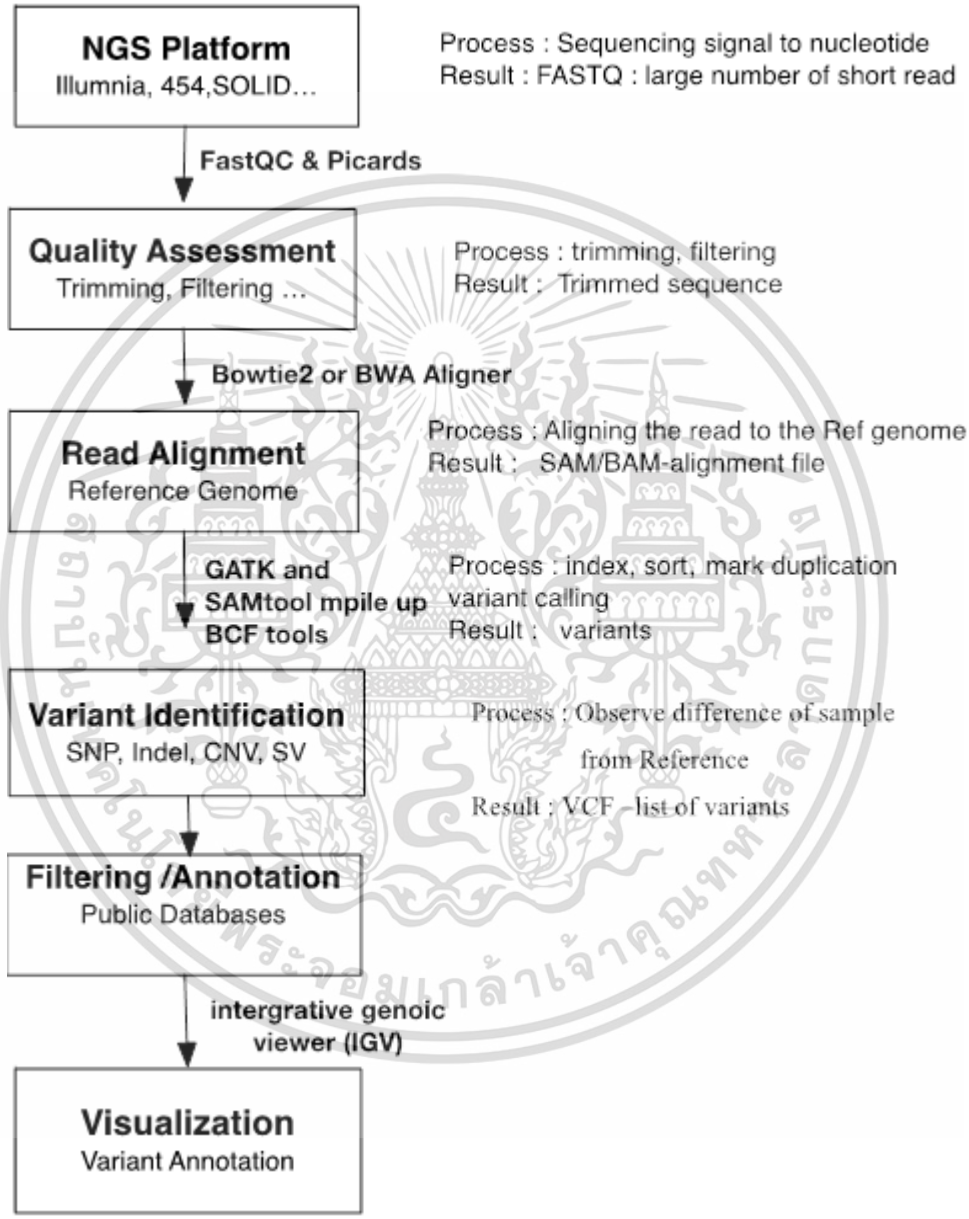


Figure 3.1 Whole genome sequencing workflow (Regmi *et al*., 2015)

3.9 Determination of expression level of putative efflux pump genes using real-time quantitative reverse transcription PCR method (Real-time qRT-PCR)

3.9.1 Primer design for cDNA synthesis and real-time PCR

Primers for cDNA synthesis (RT-primers) were designed from specific region at the C-terminal of efflux pump genes of *M. tuberculosis* H37Rv. Furthermore, real-time PCR forward and reverse primers were designed 100-bp far away from the RT-primers that could generate PCR product sizes of 200-300 bp for each gene. Primer sequences, melting temperature (Tm) and real-time PCR product sizes of efflux pump genes were shown in Table 3.3.

Table 3.3 Primers of 16 efflux pump genes, one regulator and *eis* for cDNA synthesis and real-time PCR

Genes	Primers[a]	Sequences (5′→3′)	Tm (°C)	Product sizes (bp)[b]
Rv0194	RT-Rv0194	TGTGGGCGCCGTCCTCGACA	60	
	F-Rv0194	CGATGCCACCGATGCCCAGG	59	225
	R-Rv0194	GGCCTCGGTGGCAGGATCCA	58	
Rv0783c	RT-Rv0783c	GAATGCCGCGGGGATCAGCG	60	
	F-Rv0783c	TGGGCATGGGCATGGGCTGC	60	254
	R-Rv0783c	CGAGGAAGGGTCAACCGCCG	59	
Rv1145	RT-Rv1145	TCGGGAACAGCGCAGTCGCC	61	262
	F-Rv1145	GGCGGCTCGGCGATGGAGTA	60	
	R-Rv1145	CGATAGCCAACGCGAGGCCC	60	
Rv1146	RT-Rv1146	CCATTGCGCGTCAGCGAGGC	62	256
	F-Rv1146	GCCGCCAACGACGAGAGCGT	61	
	R-Rv1146	GCTCATGCAGCCACGCCAGG	61	
Rv1250	RT-Rv1250	GCGGCTGGGTGTCGCAATCC	59	241
	F-Rv1250	ACCTGGCCGGTGCGAGCTCA	61	
	R-Rv1250	AGGGCGACGAAGGCGGGCAA	61	
Rv1258c (*tap*)	RT-Rv1258c	GAGCCGATCCTACGGGCCGA	59	226
	F-Rv1258c	GGTCATCGCGTTCCTGCCGC	60	
	R-Rv1258c	GGCATGCAGTCCAGCGGCGT	61	

Table 3.3 Primers of 16 efflux pump genes, one regulator and *eis* for cDNA synthesis and real-time PCR (continued)

Genes	Primers[a]	Sequences (5′→3′)	Tm (°C)	Product sizes (bp)[b]
Rv1410c	RT-Rv1410c	ACAGACCTAGCACCGCGCCG	61	244
	F-Rv1410c	TGCCGGCGATGCACACCGAC	60	
	R-Rv1410c	AGGCTGGCGTTGGGCGGGAT	61	
Rv1456c	RT-Rv1456c	GGCACCGGCCACGTGAATGG	58	209
	F-Rv1456c	ACGGGCACGCTAGTCACGGC	60	
	R-Rv1456c	AACCTGGCCGCGGTGCTGGT	61	
Rv1457c	RT-Rv1457c	GCCCCACACCGCTAGGACGA	58	212
	F-Rv1457c	TCGGCTTTGCGCTCGGCTGG	62	
	R-Rv1457c	CGCCGTCGGGATCACGTTCG	60	
Rv1458c	RT-Rv1458c	CTGCCGCAACTTCCTGCCGG	60	268
	F-Rv1458c	GCGATGGCGTGACCGTGGTG	59	
	R-Rv1458c	ACCTGCGGGTCAACCGGACC	60	
Rv1634	RT-Rv1634	CAACGCGGTAAGCGCCGGTC	60	
	F-Rv1634	GGTGATGGCGTCGGGGTTGG	58	251
	R-Rv1634	TGACCACCACACCGGCCAGC	58	
Rv1819c	RT-Rv1819c	TTACACTTCGGCGGGCGCCG	61	
	F-Rv1819c	CACGCTGACCAAGGTGGCGC	60	221
	R-Rv1819c	TGCAGTCCGGCAGCTCGCTG	62	
Rv1877	RT-Rv1877	GAACGAGCACCGCGTCGGTG	60	
	F-Rv1877	TGCCGAACGGGGTGCGACTG	60	261
	R-Rv1877	GGGAGTCGACCTGACGGTGC	57	
Rv2333c	RT-Rv2333c	GCGCACTCGACGGTCATCGC	61	
	F-Rv2333c	CCGCCTGGCTTTCGGCCACA	61	219
	R-Rv2333c	CAGTGGCGACGAGCAACGCC	61	
Rv2416c (*eis*)	RT-Rv2416c	CACTGGCAAACGCCGCGTCG	61	
	F-Rv2416c	GGACCCGTTACCCCACCTGC	56	224
	R-Rv2416c	CTGCCGCATCGGTCGGGGTA	60	
Rv2846c	RT-Rv2846c	ACAGCTCGCCGGCGTCGATC	62	
	F-Rv2846c	TGGCCGTCGTCCCGCTGACT	60	240
	R-Rv2846c	CCCACAGCAGGCCGTAGGTG	59	

Table 3.3 Primers of 16 efflux pump genes, one regulator and *eis* for cDNA synthesis and real-time PCR (continued)

Genes	Primers[a]	Sequences (5′→3′)	Tm (°C)	Product sizes (bp)[b]
Rv3065	RT-Rv3065	GGCACCCGCCAGGTTCAACG	58	
	F-Rv3065	CTCTTGTGCGCGATCTTCGCGG	63	210
	R-Rv3065	GGCGACCAGCACAATGGCGG	60	
Rv3197A (*whiB7*)	RT-whiB7	GCATCCTTGCGCGGACGTCC	60	
	F-whiB7	CTGACAGTCCCCAGACAGACCC	57	196
	R-whiB7	CACCACCCCAAACGCCCCAG	57	

[a] RT primers used for cDNA synthesis, F and R primers used for real-time PCR

[b] Product sizes of real-time PCR

3.9.2 Complementary DNA (cDNA) synthesis

cDNA was prepared from 100 ng RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Massachusetts, USA) and the components described in Table 3.4. The mixture was incubated at 42 °C for 30 min and the reaction was stopped at 85 °C for 5 min. The negative control of RT-PCR was performed with all components except the enzyme.

Table 3.4 Component of cDNA synthesis reaction

Components	Volumes (µl)
5X Transcriptor Reverse Transcriptase Buffer	4
Protector RNase Inhibitor (10 U/µl)	0.5
dNTPs (10 mM)	2
Transcriptor Reverse Transcriptaase (20 U/µl)	0.5
RT primer (10 µM)	4
RNA (20 ng/µl)	5
DNase free water	4
Total	**20**

3.9.3 Determination of genes expression level by real-time qRT-PCR

Efflux pump gene expression by real-time PCR was performed on PikoReal™ 24-well Real-Time PCR system as a machine (Thermo Fisher Scientific, Massachusetts, USA). The PrimeScript™ RT Master Mix (Takara, California, USA) containing PrimeScript buffer, reverse transcriptase and SYBR, was used use in this study. The component of real-time PCR reaction is shown in Table 3.5. The reaction condition consist of pre-denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 20 s and final extension at 72 °C for 10 min. The melting temperature of real-time PCR products was analyzed using temperature at 60-95 °C for 20 s. The F and R primers were shown in Table 3.3. The sigma factor *sigA* was used as control genes in this study. The relative quantification of genes expression was calculated using $2^{-\Delta\Delta Cq}$ (Putman *et al*., 2000). The expression level above 1 was considered to be increased and equal to or above 4 was considered to be overexpressed.

Table 3.5 Component of real-time PCR reactions

Components	Volumes (µl)
5X PrimeScript RT Master Mix	4
F primer (10 µM)	2
R primer (10 µM)	2
cDNA	5
RNase free water	7
Total	**20**

3.10 Functional analysis of putative efflux pump genes on AMK and KM susceptibility

3.10.1 Amplification of Rv1819c, Rv1877 and Rv2846c genes

3.10.1.1 Primer design for amplification

Primers targeted 3 putative efflux pump genes (Rv1819c, Rv1877 and Rv2846c) were designed as shown in Table 3.6. The *Bam*HI recognition sites was designed at the 5'-end of all primers for further cloning strategy. Additional primers were also designed and used as sequencing primers for sequence analysis of the amplified genes (Table 3.6).

Table 3.6 Primers for PCR amplification and sequencing of efflux pump genes

Efflux pump genes	Primers	Primer sequences (5′→3′)	Tm (˚C)	PCR products size (bp)
PCR primer				
Rv1819c	F Rv1819C-B	GGATCCTTGGGCCCGAAATTGTTTAA	58	1,932
	R Rv1819C-B	GGATCCTTACACTTCGGCGGGCGC	64	
Rv1877	F Rv1877-B	GGATCCATGGCGGGCCCCA	60	2,076
	R Rv1877-B	GGATCCCTACGTTGTAGCCGCGA	61	
Rv2846c	F Rv2846C-B	GGATCCATGACGGCTCTCAACGACAC	62	1,605
	R Rv2846C-B	GGATCCTTACAGCTCGCCGGCGTCGA	60	
Sequencing primer				
Rv1819c	F1 Rv1819C	AGGCGGCACTCCGAATGCTCC	60	-
	F2 Rv1819	CCGAGCGACGACGAGTCCGT	60	-
	R1 Rv1819C	TGATCGACTGCACGGCCCCG	60	-
	R2 Rv1819C	GCAGCGTGGTCTTGCCGGC	59.7	-
Rv1877	F1 Rv1877	CGCGGTGCTGACAGTGGCG	59.7	-
	F2 Rv1877	GTCGGGCGCCGTGCCTGT	59.4	-
	R1 Rv1877	GCCACAGCGATGACCAGGATCC	60	-
	R2 Rv1877	GGGGCGGCCATGCTCTGG	59.4	-
Rv2846c	F1 Rv2846C	CCATCGGGCGCAAACGCACC	60	-
	F2 Rv2846C	CGTCTGCATCGGCCTGTACGTG	60	-
	R1 Rv2846C	CCCTTGGGGAACGTGGTCGC	60	-
	R2 Rv2846C	5'-CTGCGAGGACACACCTAGGCC-3'	60	-

3.10.1.2 Amplification of efflux pump genes of *M. tuberculosis* by PCR method

Rv1819c, Rv1877 and Rv2846c genes of *M. tuberculosis* H37Rv were amplified with three primer pairs: F Rv1819C-B/R Rv1819C-B, F Rv1877-B/R Rv1877-B and F Rv2846C-B/R Rv2846C-B by PCR (Table 3.6). Genomic DNA of KM-resistant *M. tuberculosis* MT433 (DS no. 24433) was isolated according to the section 3.6.1.1 and used as template for PCR amplification. PCR composition was shown in Table 3.7. The PCR condition 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 60-64 °C for 1 min and extension at 72 °C for 2-2.5 min. All PCR products were purified by Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan, New Taipei) (section 3.6.3.1).

Table 3.7 PCR amplification of 3 efflux pump genes in *M. tuberculosis*

Components	Volume (µl)
10X PCR buffer	5
25 mM of $MgCl_2$	3
10 mM of dNTPs	1
5 µM of F primer	2.5
5 µM of R primer	2.5
Taq DNA polymerase (5 U/µl)	0.5
DNA template (50 ng/µl)	1
Deionized water	34.5
Total	**50 µl**

3.10.2 Ligation of PCR products into pDrive TA cloning vector

PCR products were purified as described in section 3.6.3.1 and the purified PCR products were ligated into pDrive TA cloning vector (Qiagen, Hilden, Germany). Ligation reaction was prepared as shown in Table 3.8 and incubated at 16 °C for overnight.

Table 3.8 Ligation reaction between PCR products and pDrive TA cloning vector

Components	Volume (µl)
pDrive TA cloning vector (50 ng/µl)	1
Purified PCR product (40 ng/µl)	4
2X ligation buffer containing T4 ligase (0.3 U/µl)	5
Total	**10**

3.10.3 Transformation of *E. coli*

3.10.3.1 Preparation of *E. coli* DH5α competent cells

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 for overnight with shaking at 250 rpm. Four milliliters of overnight culture was inoculated into 100 ml of SOB medium (Appendix B) in a 250-ml flask and incubated at 37 °C for 3 hrs or until OD_{600} reached 0.3-0.4. Cells were incubated on ice for 15 min and harvested by centrifugation at 5,000 xg for 15 min at 4 °C. Thirty-four mililitres of RF1 solution were added into cells and mixed gentle (Appendix B). The suspension was incubated on ice for 15 min and cells were collected by centrifugation at 5,000 xg for 15 min at 4 °C. Finally, Cells were resuspended in 4 µl of RF2 solution (Appendix B) and stored at -70 °C.

3.10.3.2 Transformation of *E. coli* DH5α by heat shock

Twenty microliters of ligation reaction as described in the section 3.10.2 were mixed with 100 µl of competent cells *E. coli* DH5α. The mixture was incubated on ice for 30 min before heating at 42 °C for 90 s. The mixture was then incubated on ice for another 2 min. After incubation, 900 µl of LB broth were added, followed by an incubation at 37 °C for 1 hr. Aliquot of 100 µl of cells was spread on LB agar containing 50 µg/ml of kanamycin, 50 µM IPTG and 80 µg/ml of X-gal for blue/white screening of recombinant clones. Plates were incubated at 37 °C overnight.

3.10.4 Analysis of recombinant plasmids by restriction enzyme digestion

The positive transformants were selected and subcultured in LB broth containing 50 µg/ml of kanamycin. Plasmid DNAs were isolated as previously

described and digested with *Eco*RI. The digestion reaction as shown in Table 3.9 was incubated at 37 ℃ for overnight. The digested products were analyzed using 0.8% (w/v) agarose gel electrophoresis (section 3.6.2.1). Subsequently, the recombinant plasmids containing expected size of DNA fragment were submitted for DNA sequencing in order to check the correct sequence of the amplified genes.

Table 3.9 Digestion reactions of recombinant plasmid pDrive carrying Rv1819c, Rv1877 and Rv2846c

Components	Volumes (µl)
Recombinant plasmid DNA (60 ng/µl)	3
*Eco*RI (12 U/µl)	1
NEBuffer 3.1 (10X)	1
BSA (0.1 mg/ml)	1
Deionized water	4
Total	**10**

3.10.5 Cloning of shuttle vector pSMT1 containing efflux pump genes

The pSMT1 is a hygromycin-resistant shuttle vector containing 2 origins of replication region for mycobacteria and *E. coli*, namely ALori and Eori (Appendix A). Expression of the interested gene in pSMT1 is under the control of BCG *hsp60* promoter (Phsp60) (Snewin *et al*., 1999).

3.10.5.1 Isolation of DNA fragment containing efflux pump genes

Recombinant plasmids pDrive containing Rv1819c, Rv1877, or Rv2846c and shuttle vector pSMT1 were digested with *Bam*HI with components as described in Table 3.10. The digestion reaction was incubated at 37 ˚C for overnight. Then, the digested products were analyzed using 0.8% (w/v) agarose gel electrophoresis. The DNA fragments of vector and insert DNAs were purified from agarose gel using the Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan).

Table 3.10 Digestion reaction of recombinant plasmid pDrive and pSMT1 vector

Components	Volumes (µl)	
	pDrive-efflux pump genes	pSMT1
pDrive-efflux pump genes (50 ng/µl)	30	-
pSMT1 shuttle vector (50 ng/µl)	-	30
*Bam*HI (12 U/µl)	5	5
NEBuffer 3.1 (10X)	5	5
BSA (0.1 mg/ml)	5	5
Deionized water	5	5
Total	**50**	**50**

3.10.5.2 Ligation and Transformation

Before ligation, the vector DNA, pSMT1 digested with *Bam*HI, was dephosphorylated using a calf intestine alkaline phosphatase (CIAP). The reaction mixture was shown in Table 3.11. After that, DNA fragments of purified Rv1819c, Rv1877 and Rv2846c were ligated to the purified pSMT1 shuttle vector with the composition shown in Table 3.12. The ligation reaction was incubated at 16 °C for overnight and transformed into competent cells *E. coli* DH5α as mentioned before, excepting that the transformants were selected on LB ager containing 75 µg/ml of hygromycin.

Table 3.11 Dephoshorylation reaction

Components	Volume (µl)
pSMT1 shuttle vector/*Bam*HI (30 ng/µl)	18
Calf intestine alkaline phosphatase enzyme (0.01 U/µl)	4
CIAP buffer (10X)	4
Deionized water	14
Total	**40**

Table 3.12 Ligation reaction of efflux pump genes and pSMT1 shuttle vector

Components	Volume (µl)
Purified DNA fragments/*Bam*HI (50 ng/µl)	6
Purified pSMT1/*Bam*HI/Dephosphorelated (50 ng/µl)	4
T4 DNA ligase (30 U/µl)	2
Ligation buffer (10X)	2
Deionized water	6
Total	**20**

3.10.5.3 Analysis of recombinant pSMT1 clones

Transformants with recombinant pSMT1 clones (Rv1819c, Rv1877 and Rv2846c) were picked up and cultured on LB agar supplemented with 75 µg/ml of hygromycin. The recombinant plasmids of these transformants were isolated. The direction of gene insertion (sense (S) and antisense (AS) directions) was determined by digestion of the recombinant plasmids with restriction enzyme *Nru*I. The digested products were analyzed using 0.8% (w/v) agarose gel electrophoresis. The component of digestion reaction was shown in Table 3.13 and the mixture was incubated at 37 ˚C for overnight. The sizes of expected *Nru*I digested products both sense (S) and antisense (AS) directions were shown in Table 3.14.

Table 3.13 Digestion reaction of recombinant pSMT1 for direction checking

Components	Volume (µl)
Purified recombinant pSMT1 (50 ng/µl)	6
*Nru*I (10 U/µl)	2
NEBuffer 3 (10X)	2
Deionized water	10
Total	**20**

Table 3.14 Size of expected *Nru*I digested products for direction analysis of recombinant pSMT1 clones

Recombinant plasmids	Size of products (bp) Sense direction	Size of products (bp) Antisense direction
pSMT1-Rv1819c	4,862, 1,775, 282	3,142, 2,002, 1,775
pSMT1-Rv1877	4,009, 1,775, 1,279	4,139, 1,775, 1,149
pSMT1-Rv2846c	3,587, 1,775, 1,230	4,090, 1,775, 727

3.10.6 Functional analysis of *M. tuberculosis* Rv1819c, Rv1877 and Rv2846c

3.10.6.1 Preparation of *M. tuberculosis* H37Ra competent cells

Competent cells of *M. tuberculosis* H37Ra were prepared by inoculating a single colony of *M. tuberculosis* H37Ra into 20 ml of Middlebrook 7H9 supplemented with 0.05% (v/v) Tween 80 and incubating at 37 °C with shaking speed at 200 rpm for 24 h. Ten mililitres of pre-culture was inoculated into 200 ml of Middlebrook 7H9 and incubated at 37 °C with shaking for 16 h or until an OD_{600} reached 0.5-1.0. After incubation on ice for 1.5 h, cells were harvested by centrifugation at 5,000 x g for 10 min at 4 °C and washed three times in ice-cold 10% (v/v) glycerol. Finally, cells were resuspended in 1:100 original culture volume of ice-cold 10% (v/v) glycerol. The competent cells should be freshly prepared before use.

3.10.6.2 Transformation of *M. tuberculosis* H37Ra by electroporation

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 is placed on electroporation chamber and subjected to one single pulse of 2.5 kV and 25 µF with the pulse-controller resistance setting at 1,000 ohms. After electroporation, the cuvette was stood on ice for 10 min and added with 1 ml of Middlebrook 7H9. The suspension is then transferred to a sterile 15 ml tube and incubated at 37 °C for 2 h. A total 200 µl of culture was spread on Middlebrook 7H11 containing 50 µg/ml of hygromycin and plates were incubated at 37 °C for 3-4 weeks.

3.10.6.3 Determination of minimal inhibitory concentration (MIC)

The analysis of relationship between efflux pump genes and aminoglycosides resistance mechanisms was performed by the determination of MIC. MICs of amikacin (AMK), kanamycin (KM) and capreomycin (CAP) were performed at final concentrations of 0, 2, 4, 8, 16, 32 and 64 µg/ml of each antibiotic. The agar dilution method was used as described in section 3.6.4.2. The contamination of culture was determined visually and the result was checked after incubation for 3-4 weeks. The MIC is defined as the lowest concentration of an antibiotic that inhibits 99% of microbial growth.

CHAPTER 4

Main Results

4.1 Genetic characterization of amikacin (AMK), kanamycin (KM) and capreomycin (CAP) resistance in KM-resistant *M. tuberculosis* clinical strains isolated from Thai patients

In this study, nucleotide sequencing of genes associated with aminoglycoside and capreomycin resistance in KM-resistant and KM-susceptible *M. tuberculosis* clinical isolates from Thai patients was determined. The genes that has been previously reported to associate with aminoglycoside and capreomycin resistance in *M. tuberculosis* include *rrs*, *eis* including promoter region, *tap*, *whiB7* and *tlyA.* They were amplified by using genomic DNA of KM-resistant and KM-susceptible *M. tuberculosis* clinical isolates as templates and their PCR products were sequenced. Then, nucleotide sequences of all PCR products were aligned with genome sequence of *M. tuberculosis* H37Rv (Accession no. NC_000962.3) using ClustalW program.

4.1.1 Genomic DNA isolation of KM-resistant and -susceptible *M. tuberculosis* clinical isolates

The 29 KM-resistant and 27 KM-susceptible *M. tuberculosis* isolates were grown on Middlebrook 7H10 agar as described in 3.5.1. Genomic DNAs were isolated according to the protocol as described in section 3.6.1.1 and subsequently were analysed by 0.8% (w/v) agarose gel electrophoresis and spectrophotometric method. Figure 4.1 shows the example of genomic DNAs isolated from KM-resistant and KM-susceptible *M. tuberculosis* strains. The result showed that only one DNA band was appeared in each genomic DNA (Fig. 4.1) and the ratio of A_{260}/A_{280} of all isolated genomic DNAs was approximately 1.8 indicating that their genomic DNAs had a good quality. The concentration of genomic DNAs estimated 50 ng/µl. The genomic DNA was subsequently used for PCR amplification of genes involving in aminoglycoside and capreomycin resistance.

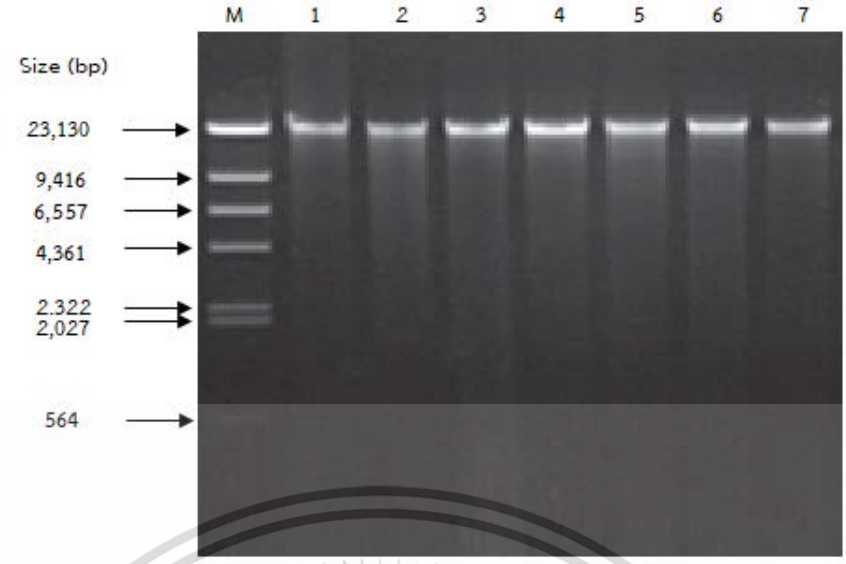


Figure 4.1 Genomic DNAs of KM-resistant and –susceptible *M. tuberculosis* strains

Lane M: λ DNA/*Hin*dIII fragments marker

Lane 1 : Genomis DNA of KM-resistant *M. tuberculosis* MT111

Lane 2 : Genomis DNA of KM-resistant *M. tuberculosis* MT120

Lane 3 : Genomis DNA of KM-susceptible *M. tuberculosis* MT127

Lane 4 : Genomis DNA of KM-susceptible *M. tuberculosis* MT164

Lane 5 : Genomis DNA of KM-resistant *M. tuberculosis* DS004

Lane 6 : Genomis DNA of KM-resistant *M. tuberculosis* DS016

Lane 7 : Genomis DNA of KM-susceptible *M. tuberculosis* DS039

4.1.2 DNA amplification of genes associated with resistance of AMK, KM and CAP in *M. tuberculosis* by PCR

Primers were designed based on the published genome sequence of *M. tuberculosis* H37Rv from Genbank (accession no. NC_000962). Nucleotide sequences of all primers (forward, reverse and sequencing primers) and the expected PCR product sizes were shown in Table 3.1. The *rrs*, *eis* including promoter region, *tap*, *whiB7* and *tlyA* were amplified by PCR. After amplification, the PCR products were analysed using 0.8% (w/v) agarose gel electrophoresis. The PCR products of *rrs*, *eis* including promoter region, *tap*, *whiB7* and *tlyA* showed the correct sizes with 1680, 1660, 1847, 550 and 807 bp, respectively (Fig. 4.2), indicating that all primer designed in this study were specific to the corresponding genes. In addition, no PCR product of

all genes was found in the negative control using deionized water instead of genomic DNA as a template. Before sequencing, the PCR products was purified by Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan). The concentration of the purified PCR products was approximately 20-100 ng/µl estimated by agarose gel electrophoresis.

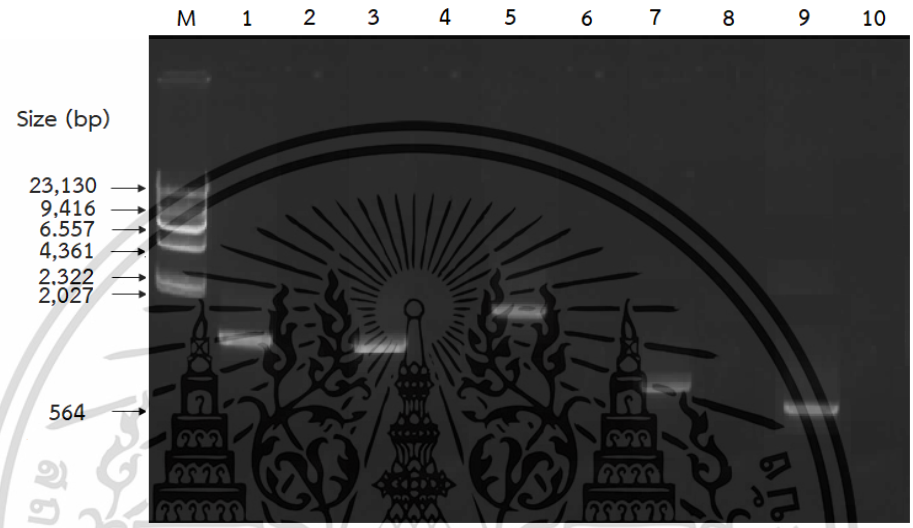


Figure 4.2 PCR products of *rrs*, *eis*, *tap*, *tlyA* and *whiB7* of *M. tuberculosis* MT433

Lane M : λ DNA/*Hin*dIII fragments marker

Lane 1 : PCR product of *rrs*

Lane 2 : Negative PCR product of *rrs*

Lane 3 : PCR product of *eis* including promoter

Lane 4 : Negative PCR product of *eis* including promoter

Lane 5 : PCR product of *tap*

Lane 6 : Negative PCR product of *tap*

Lane 7 : PCR product of *tlyA*

Lane 8 : Negative PCR product of *tlyA*

Lane 9 : PCR product of *whiB7*

Lane 10: Negative PCR product of *whiB7*

4.1.3 Analysis of *rrs*, *eis* including promoter region, *tap*, *whiB7* and *tlyA* nucleotide sequences

PCR products of *rrs*, *eis* including promoter region, *tap*, *whiB7* and *tlyA* genes amplified from 29 KM-resistant and 27 KM-susceptible *M. tuberculosis* isolates were submitted for nucleotide sequencing at First BASE Laboratories (Malaysia) using forward, reverse and sequencing primers. Nucleotide sequences of five genes were analysed from ABI PRISM^{R} 3700 DNA analyser. The examples of sequencing chromatogram of each gene were shown in Appendix C. All obtained nucleotide sequences of each gene were compared with those of genome sequence of *M. tuberculosis* H37Rv by ClustalW program. The results of sequence alignment of all five genes in KM-resistant and KM-susceptible *M. tuberculosis* isolates are shown in Table 4.1 and Table 4.2, respectively. In addition, MIC values of both KM-resistant and KM-susceptible isolates are also shown in Table 4.1 and Table 4.2. Considering the *rrs* or 16S rRNA gene, most often reported gene involving in the resistance of aminoglycosides, the point mutation was found in 21 KM-resistant *M. tuberculosis* isolates whereas no *rrs* mutation was found in only eight KM-resistant *M. tuberculosis* isolates Table 4.1. In all 27 KM-susceptible *M. tuberculosis* isolates, no *rrs* mutation was shown (Table 4.2). In 21 KM-resistant *M. tuberculosis* isolates with *rrs* mutation, the alteration of adenine to guanine at the position 1401 (A1401G) of *rrs* was only found (Table 4.1).

Table 4.1 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 29 KM-resistant clinical isolates of *M. tuberculosis*

Isolate (DS no.)	Drug resistance	Genotype	MIC (μg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
			AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
MT012 (17012)	XDR	NBJ	>64	>64	16	A1401G	wt	wt	wt	wt	A33G	wt
MT016 (17016)	XDR	BJ	>64	>64	16	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT058 (27058)	XDR	BJ	>64	>64	16	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT092 (17092)	XDR	BJ	>64	>64	4	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT104 (22104)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT109 (19109)	XDR	BJ	>64	>64	>64	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT111 (19111)	XDR	NBJ	>64	>64	32	A1401G	wt	wt	wt	wt	A33G	wt

Table 4.1 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 29 KM-resistant clinical isolates of *M. tuberculosis* (continued)

Isolate (DS no.)	Drug resistance	Genotype	MIC (μg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
			AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
MT120 (20120)	XDR	BJ	8	>64	>64	wt	C-14T	Ins581C	Frameshift (419>231)	wt	A33G Ins49GC	wt Frameshift (268>26)
MT127 (21127)	XDR	NBJ	>64	>64	32	A1401G	wt	wt	Frameshift (419>231)	wt	A33G	wt
MT164 (17164)	XDR	NA	>64	>64	16	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT179 (16179)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT182 (18182)	XDR	NBJ	>64	>64	16	A1401G	wt	wt	Frameshift (419>231)	wt	A33G	wt
MT260 (14260)	XDR	BJ	8	>64	>64	wt	C-14T	Ins581C	Frameshift (419>231)	wt	A33G T539G	wt L180R
MT287 (13287)	XDR	NBJ	>64	>64	32	A1401G	wt	wt	wt	wt	A33G	wt
MT381 (19381)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt

Table 4.1 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 29 KM-resistant clinical isolates of *M. tuberculosis* (continued)

Isolate (DS no.)	Drug resistance	Genotype	MIC (μg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
			AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
MT388 (21388)	XDR	BJ	>64	>64	16	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT515 (18515)	XDR	NBJ	>64	>64	32	A1401G	wt	wt	wt	wt	A33G	wt
MT592 (17592)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT653 (17653)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT688 (17688)	XDR	BJ	8	32	8	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT810 (18810)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT825 (16825)	XDR	BJ	>64	>64	16	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT848 (21848)	XDR	NBJ	>64	>64	32	A1401G	wt	wt	wt	wt	A33G	wt

Table 4.1 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 29 K resistant clinical isolates of *M. tuberculosis* (continued)

Isolate (DS no.)	Drug resistance	Genotype	MIC (µg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
			AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
MT962 (17962)	XDR	BJ	>64	>64	32	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT966 (15966)	XDR	BJ	8	>64	>64	wt	G-37T	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT984 (17984)	XDR	BJ	8	>64	8	wt	C-14T	Ins581C	Frameshift (419>231)	wt	A33G	wt
MT433 (24433)	Pre-XDR	BJ	>64	>64	16	wt	wt	wt	wt	wt	A33G	wt
MT617 (13617)	Pre-XDR	BJ	8	>64	>64	wt	C-14T	Ins581C	Frameshift (419>231)	wt	A33G T539G	wt L180R
MT845 (17845)	Pre-XDR	BJ	>64	>64	16	A1401G	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt

wt, wild-type; NM, No mutation; MDR, Multidrug resistance; XDR, Extensively drug resistance; BJ, Beijing; NBJ, Non-Beijing; NA, Not analyzed; C-14T, Nucleotide change at position -14 from cytosine to thymine; G-37T, Nucleotide change at position -37 from guanine to thymine; Ins581C, Insertion of cytosine at position 581; A33G, Nucleotide change at position 33 from adenine to guanine; Ins49GC, Nucleotide change at position 49 by insertion of guanine and cytosine; T539G, Nucleotide change at position 539 from thymine to guanine; L180R, Amino acid change at codon 180 from leucine to arginine; Frameshift (419>231) and frameshift (268>26), Mutation caused size reduction from 419 or 268 to 231 or 26, respectively

Table 4.2 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 27 AMK- and KM-susceptible clinical isolates of *M. tuberculosis*

Isolate (DS no.)	MIC (μg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
	AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
DS004 (23004)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS016 (27016)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS039 (26039)	2-4	4	2-4	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
DS165 (27165)	2-4	4	2-4	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
DS280 (10280)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS305 (10305)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS307 (10307)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS320 (10320)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS378 (10378)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS379 (10379)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS386 (9386)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt

Table 4.2 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin ir AMK- and KM-susceptible clinical isolates of *M. tuberculosis* (continued)

Isolate (DS no.)	MIC (µg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
	AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
DS394 (9394)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS563 (21563)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS596 (9596)	2-4	4	2-4	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
DS644 (21644)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS684 (23684)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS706 (21706)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS722 (8722)	2-4	4	2-4	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
DS736 (20736)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS747 (9747)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS812 (9812)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS851 (9851)	2	4	4	wt	wt	wt	wt	wt	A33G	wt

Table 4.2 Genetic characterization of genes associated with KM resistance and MIC values for amikacin, kanamycin and capreomycin in 27 AMK- and KM-susceptible clinical isolates of *M. tuberculosis* (continued)

Isolate (DS no.)	MIC (μg/ml)			*rrs* mutation	*eis* promoter mutation	*tap* mutation		*whiB7* promoter mutation	*tlyA* mutation	
	AK	KM	CAP			Nucleotide change	Amino acid change		Nucleotide change	Amino acid change
DS859 (21859)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS874 (9874)	2-4	4	2-4	wt	wt	Ins581C	Frameshift (419>231)	wt	A33G	wt
DS877 (9877)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS945 (21945)	2-4	4	2-4	wt	wt	wt	wt	wt	A33G	wt
DS1945 (DS21945)	2-4	4	2	wt	wt	wt	wt	wt	A33G	wt
H37Rv	2	4	4	wt	wt	wt	wt	wt	wt	wt

wt, wild-type; Ins581C, Insertion of cytosine at position 581; A33G, Nucleotide change at position 33 from adenine to guanine; Frameshift (419>231), Mutation caused size reduction from 419 to 231 residues

Figure 4.3 shows a point *rrs* mutation at A1401G of KM-resistant *M. tuberculosis* MT287 compared with the *rrs* sequence of *M. tuberculosis* H37Rv. All *M. tuberculosis* isolates with A1401G mutation showed a high-level of resistance to both KM and AMK with minimum inhibitory concentration (MIC) >64 µg/ml whereas they showed a broad range of CAP resistance with MIC from 4 to >64 µg/ml (Table 4.1). In addition, eight KM-resistant *M. tuberculosis* isolates without A1401G *rrs* mutation showed a high-level resistance to KM (>64 µg/ml) (Table 4.1). In KM-resistant *M. tuberculosis* isolates without A1401G *rrs* mutation, other genes are suggested to associate with aminoglycosides resistance.

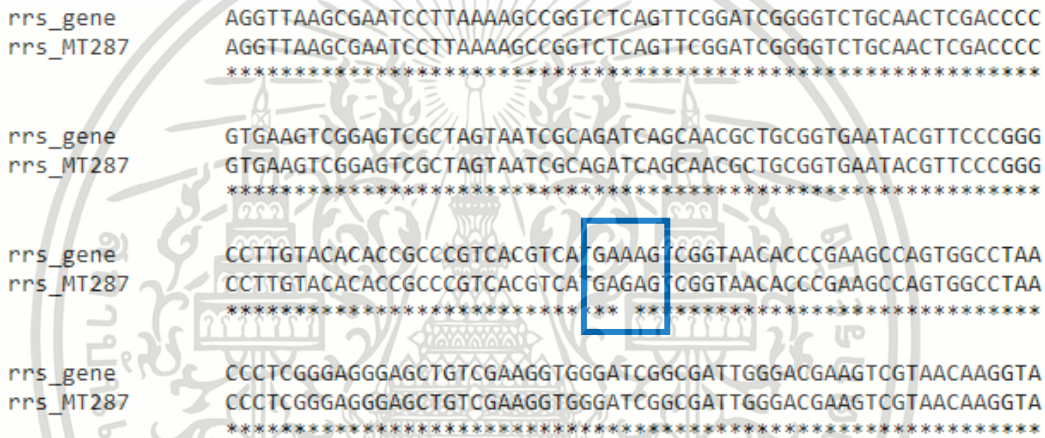


Figure 4.3 Alignment of *rrs* nucleotide sequence of KM-resistant *M. tuberculosis* MT287 compared with that of *M. tuberculosis* H37Rv. A blue square box shows A1401G mutation position in *M. tuberculosis* MT287.

In eight KM-resistant *M. tuberculosis* isolates without A1401G *rrs* mutation, *eis* including promoter region, *tap*, *whiB7* and *tlyA* genes were amplified by PCR and their PCR products were sequenced. The C-14T (cytosine to thymine at position -14) *eis* mutation was found in four KM-resistant strains without *rrs* mutation (MT120, MT260, MT617 and MT984) and the G-37T (guanine to thymine at position -37) *eis* mutation was observed in only one KM-resistant strain without *rrs* mutation (MT966) (Table 4.1). Figure 4.4 shows C-14T and G-37T point mutations at promoter region of *eis* gene of KM-resistant *M. tuberculosis* MT260 and MT966, respectively, compared with the *eis* sequence of *M. tuberculosis* H37Rv. PCR amplification and nucleotide

sequencing of *eis* including promoter region was also investigated in 27 KM-susceptible *M. tuberculosis* isolates. From sequence analysis, no mutation of *eis* including promoter region were found in all KM-susceptible isolates in this study (Table 4.2).

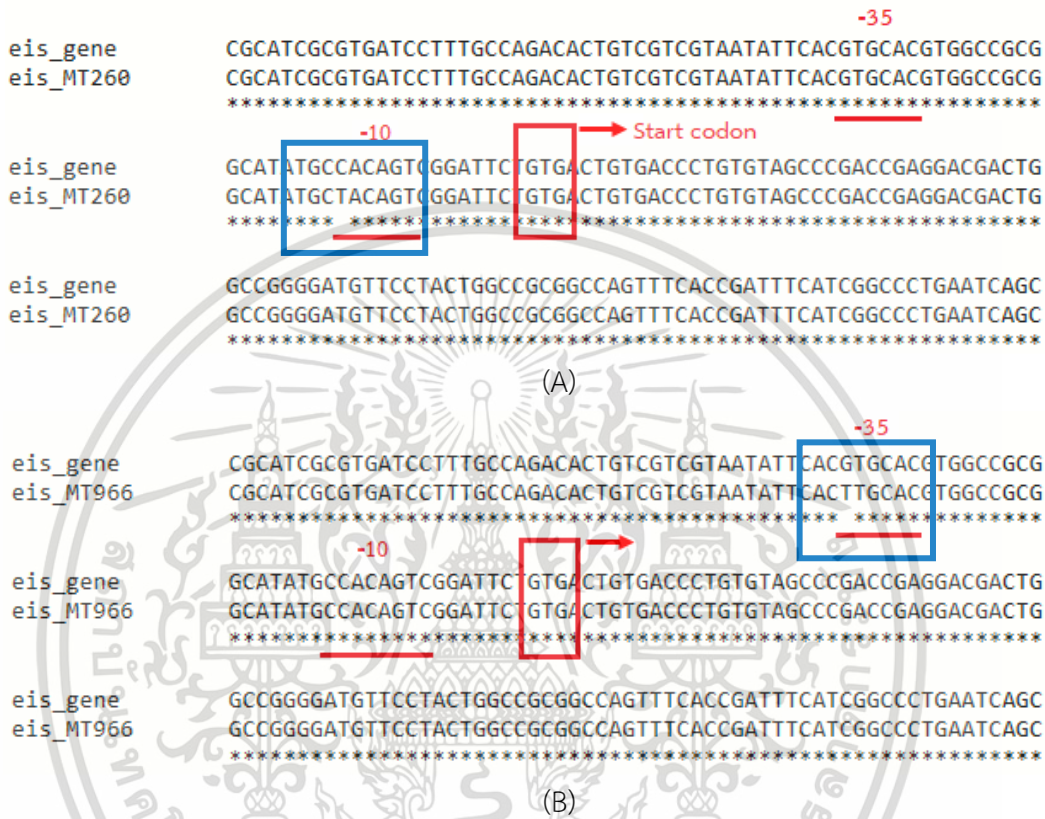


Figure 4.4 Alignment of nucleotide sequence at *eis* promoter region of KM-resistant *M. tuberculosis* MT260 (A) and *M. tuberculosis* MT966 (B) compared with that of *M. tuberculosis* H37Rv. A blue square box shows C-14T (A) and B-37T (B) mutations.

In this study, the nucleotide sequence analysis of *tap* was investigated in eight KM-resistant *M. tuberculosis* strains without *rrs* mutation and eight representative KM-susceptible *M. tuberculosis* strains. The result showed that the insertion of cytosine between positions 580 and 581 (Ins581C) was found in seven KM-resistant strains without *rrs* mutation and 6 KM-susceptible strains (Table 4.1 and Table 4.2). Only one KM-resistant *M. tuberculosis* MT433 and two KM-susceptible *M. tuberculosis* DS394 and DS1945 showed neither point mutation nor insertional mutation (Table 4.1 and Table 4.2). Figure 4.5 shows Ins581C mutation of *tap* gene of

KM-resistant *M. tuberculosis* MT966, compared with the *tap* sequence of *M. tuberculosis* H37Rv. The Ins581C mutation causes a premature stop codon resulting in the reduction of Tap sizes from 419 amino acids to 231 amino acids, finally affecting the function of Tap.

Figure 4.5 Alignment of *tap* nucleotide sequence of KM-resistant *M. tuberculosis* MT966 comparing with genomic DNA of *M. tuberculosis* H37Rv. A blue square box shows insertion of cytosine at position 581 of *tap* gene.

The nucleotide sequence analysis of 5'-untranslated region of transcriptional regulator *whiB7* was also investigated in eight KM-resistant *M. tuberculosis* isolates without A1401G *rrs* mutation and 27 KM-susceptible *M. tuberculosis* isolates. It was found that no mutation of *whiB7* was shown in both KM-resistant and KM–susceptible strains (Table 4.1 and Table 4.2). Nucleotide sequence of 5'-UTR of *whiB7* in KM-resistant *M. tuberculosis* MT433 compared with genomic DNA of *M. tuberculosis* H37Rv was shown in Fig. 4.6.

From nucleotide sequence analysis of *tlyA* in all 29 KM-resistant and 27 KM–susceptible *M. tuberculosis* strains, all strains exhibited the nucleotide alteration in *tlyA* at the position 33 (from adenine to guanine or A33G) (Table 4.1 and Table 4.2). It was suggested that A33G might be polymorphism for Beijing strain. Besides A33G mutation found in all strains, two additional *tlyA* mutations were found in three KM-resistant strains; an insertion of GC at position 49 (Ins49GC) was found in *M. tuberculosis* MT120 and nucleotide alteration at position 539 (thymine to guanine

(T539G)) was found in *M. tuberculosis* MT260 and *M. tuberculosis* MT617 (Table 4.1 and Table 4.2).

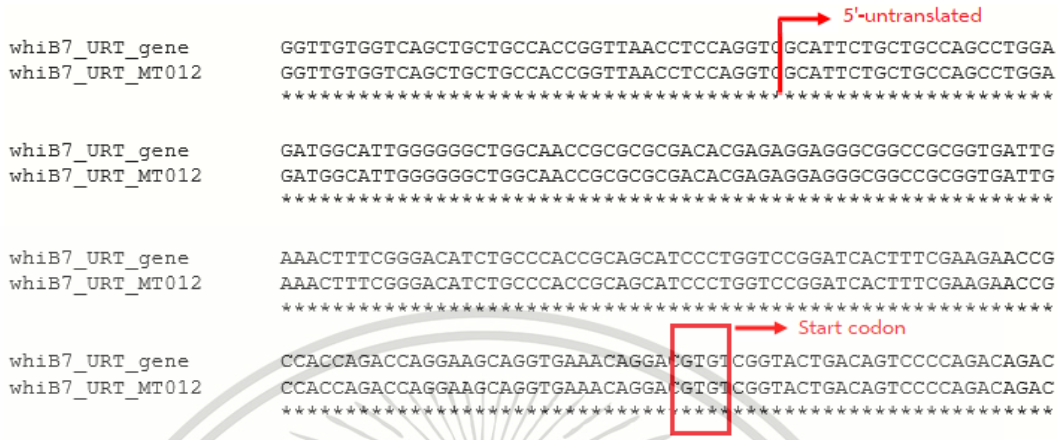


Figure 4.6 Alignment of *whiB7* nucleotide sequence of KM-resistant *M. tuberculosis* MT433 comparing with genomic DNA of *M. tuberculosis* H37Rv.

Figure 4.7 shows A33G, Ins49GC and T539G mutations of *tlyA* gene of KM-resistant *M. tuberculosis* MT092, MT120 and MT260, respectively, compared with the *tap* sequence of *M. tuberculosis* H37Rv. The Ins49GC mutation of *tlyA* caused a frameshift mutation, leading to the reduction of TlyA size from 268 amino acids to 26 amino acids. The T539G mutation of *tlyA* caused amino acid change from lysine (L) to arginine (R) at the codon 180. The results of MIC and molecular characterization of genes associated with aminoglycoside resistance in 29 KM-resistant *M. tuberculosis* strains and 27 KM-susceptible *M. tuberculosis* strains are summarized in Table 4.3. Among KM-resistant strains, 21 strains showed A1401 *rrs* mutation whereas eight strains showed no *rrs* mutation. Twenty KM-resistant strains with A1401G *rrs* mutation showed a high-level resistance to AMK and KM (MIC >64 µg/ml) but one KM-resistant strain with A1401G *rrs* mutation showed a lower MIC to AMK and KM than other A1401G *rrs* mutant strains. Among eight non *rrs* mutant strains, C-14T and G-37T *eis* promoter mutation were detected in five strains. Interestingly, all strains with *eis* promoter mutations showed a high-level resistance to KM (MIC >64 µg/ml). Besides *eis* promoter mutation, these five strains contained also Ins581C *tap* mutation. However, Ins581C tap mutation might be not directly

involved in KM resistance due to the existence of this point mutation in five KM-susceptible strains. In addition, all KM-resistant and KM–susceptible *M. tuberculosis* clinical strains showed a polymorphism mutation in *tlyA* (A33G) but two different point mutations were found in three KM-resistant strains. Interestingly, only one strain (*M. tuberculosis* MT433) from twenty-nine KM-resistant strains did not exhibit any mutations in all aminoglycoside-resistant genes. It is suggested that there is other resistance mechanism or a novel point gene mutation in this KM-resistant *M. tuberculosis* strain.

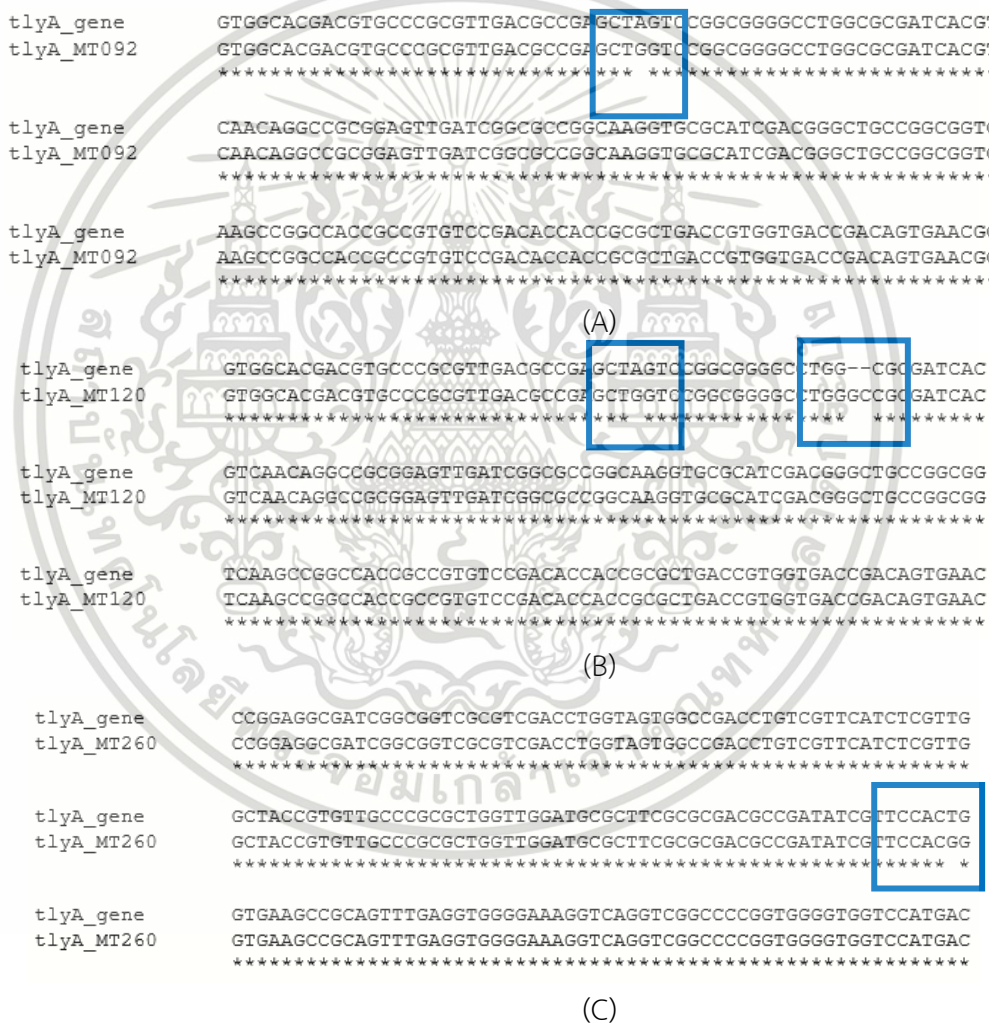


Figure 4.7 Alignment of *tlyA* nucleotide sequence of KM-resistant *M. tuberculosis* MT092 (A), MT120 (B) and MT260 (C) compared with genomic DNA of *M. tuberculosis* H37Rv. The blue square boxes show A33G (A), Ins49GC (B) and T539G (C) mutations of *tlyA*.

Table 4.3 Sequence analysis of *eis* promoter region, *tap*, *whiB7* and *tlyA* genes and MIC values for AK, KM and CAP of 29 KM-resistant *M. tuberculosis* clinical isolates

No. of strains	MIC (µg/ml)			Gene / Mutation				
	AMK	KM	CAP	*rrs*	*eis*	*tap*	*whiB7*	*tlyA*
KM resistant (29)								
1	>64	>64	>64	A1401G	wt	Ins581C	wt	A33G[b]
7	>64	>64	32	A1401G	wt	Ins581C	wt	A33G[b]
5	>64	>64	32	A1401G	wt	wt	wt	A33G[b]
4[a]	>64	>64	16	A1401G	wt	Ins581C	wt	A33G[b]
2	>64	>64	16	A1401G	wt	wt	wt	A33G[b]
1	>64	>64	4	A1401G	wt	Ins581C	wt	A33G[b]
1	8	32	8	A1401G	C-14T	Ins581C	wt	A33G[b]
1	8	>64	8	wt	C-14T	Ins581C	wt	A33G[b]
1	8	>64	>64	wt	C-14T	Ins581C	wt	A33G[b] /Ins49GC
2[a]	8	>64	>64	wt	C-14T	Ins581C	wt	A33G[b] /T539G
1	8	>64	>64	wt	G-37T	Ins581C	wt	A33G[b]
2	>64	>64	16	wt	wt	Ins581C	wt	A33G[b]
1[a]	>64	>64	16	wt	wt	wt	wt	A33G[b]
KM susceptible (27)								
5	2-4	4	2-4	wt	wt	Ins581C	wt	A33G[b]
22	2-4	4	2-4	wt	wt	wt	wt	A33G[b]

[a] include one MDR-TB strain; Ins, insertion; wt, wild type; NA, not analyzed

[b] no amino acid chang

4.2 Analysis of genes involving in aminoglycoside resistance including efflux pump in AMK- and KM-resistant *M. tuberculosis* MT433 by whole genome sequencing (WGS)

From the results of genetic characterization of *rrs*, *eis* promoter region, 5'-untranslated of *whiB7*, *tap* and *tlyA* of 29 KM-resistant *M. tuberculosis* clinical strains, it was shown that only AMK- and KM-resistant *M. tuberculosis* MT433 did not contain any mutations in genes associated with aminoglycoside resistance reported (Table 4.3). It is possible that there is an unknown or a novel mechanism of aminoglycoside resistance or there are other point mutations of involving genes in this strain. Therefore, whole genome sequencing of *M. tuberculosis* MT433 was determined on an Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA). The obtained whole genome sequences were deposited in GenBank under accession no. LGX00000000 and were compared with *M. tuberculosis* H37Rv reference genome (GenBank accession number NC_000962.3). In this study, the 40,836,734 paired-end reads were generated with an average length of 100 bp and approximately 99% of reads were aligned to the reference genome. The coverage was investigated by bedtools (version 17.2.0) (a power toolset for genome arithmetic), resulting in approximately 99% of coverage with reference genome. The result of genome assembly showed 4,409,112 bp with 285 contigs and contigs had an average length of 15,470 bp and average coverage of 595X. The GC content of genome of *M. tuberculosis* MT433 was 65.17%. In addition, the genome annotation analysis was performed by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The result of genome annotation of *M. tuberculosis* MT433 strain showed 4,172 genes, 4,084 coding sequences, 35 pseudogenes and 53 structural RNAs including 45 tRNAs, 3 rRNAs and 5 noncoding RNAs. The single-nucleotide polymorphisms (SNPs) were investigated by the Genome analysis tool kit (GATK) (version 3.2) using SnpEff programme (genomic variant annotations and functional effect prediction toolbox). In *M. tuberculosis* MT433, 833 SNPs comprising 342 nonsymnonymous, 218 synonymous and 273 intergenic SNPs are shown in Appendix D.

In this study, the WGS result of *M. tuberculosis* MT433 genome confirmed the absence of point mutations in aminoglycoside-resistant genes including *rrs*, *tap*, *tlyA* and *eis* promoter. It is speculated that aminoglycoside resistance mechanism of

M. tuberculosis MT433 is involved in the efflux pump mechanism. From WGS data of *M. tuberculosis* MT433, nucleotide sequence of a total of sixteen efflux pump genes and hypothetical transmembrane genes was determined. The result showed that only two genes, Rv0194 and Rv1250, showed an alteration of nucleotide sequence (Table 4.4). In Rv0194, multidrug efflux ATP-binding/permease protein, thymine at position 221 was changed to cytosine (T221C), resulting in the amino acid change from methionine to threonine at codon 74 (M74T). The point mutation in Rv0194 leads to the non-synonymous mutation (Table 4.4). Another nucleotide change was found in Rv1250, MFS family transporter. In Rv1250, cytosine at position 502 was changed to thymine (C502T) but this mutation did not make any amino acid change, thus it might not be conferred KM resistance in this strain (Table 4.4).

Table 4.4 Analysis of mutation in 16 efflux pump or hypothetical transmembrane gene in *M. tuberculosis* MT433

Genes	Function or predicted function (drug resistance)	Nucleotide change	Amino acid change	Synonymous or non-synonymous
Rv0194	VAN and TET	T221C	M74T	Non Syn
Rv0783c	FQs	wt	wt	wt
Rv1145	Multidrug resistance pump	wt	wt	wt
Rv1146	Multidrug resistance pump	wt	wt	wt
Rv1250	INH	C502T	wt	Syn
Rv1258c	TET and AGs	wt	wt	wt
Rv1410c	TET and AGs	wt	wt	wt
Rv1456c	INH, RIF, EMB and STR	wt	wt	wt
Rv1457c	INH, RIF, EMB and STR	wt	wt	wt
Rv1458c	INH, RIF, EMB and STR	wt	wt	wt
Rv1634	Multidrug resistance pump	wt	wt	wt
Rv1819c	INH	wt	wt	wt
Rv1877	FQs and AGs	wt	wt	wt
Rv2333c	SPE and TET	wt	wt	wt
Rv2846c	INH, RIF and ERY	wt	wt	wt
Rv3065	ERY	wt	wt	wt

VAN, vanomycin; TET, tetracycline; FQs, fluoroquinolones; INH, isoniazid; AGs, aminoglycosides; RIF, rifampicin; EMB, ethambutol; STR, streptomycin; SPE, spectinomycin; ERY, erythromycin

4.3 Study of gene expression level of putative efflux pump genes using real-time quantitative reverse transcription PCR method (Real-time qRT-PCR)

Apart from the nucleotide change, the level of gene expression of efflux pump or hypothetical transmembrane genes might play a role in aminoglycoside resistance mechanism. In this study, the expression levels of sixteen efflux pump genes, hypothetical predicted transmembrane proteins encoding genes, and transcriptional regulator *whiB7* (Rv3197c) and aminoglycosides modifying enzymes (acetyltransferases) encoding *eis* (Rv2416c), were determined in KM-resistant *M. tuberculosis* MT433 (strain with no mutation in all aminoglycoside-resistant genes) and MT164 (strain containing A1401G *rrs* mutation) by real time qRT-PCR. Total RNA of both strains was isolated and reverse-transcribed into complementary DNA (cDNA) by reverse transcription method. The obtained cDNA was used as a template for analysis of those gene expression levels. Finally, genes expressed at high level were selected for study the function involving in aminoglycoside resistance.

4.3.1 Total RNA extraction of *M. tuberculosis* MT433 and MT164

M. tuberculosis MT433 and MT164 were grown on Middlebrook 7H10 agar (Becton Dickinson, New Jersey, USA) at 37 °C for 3-4 weeks (section 3.5.1). Cells of both strains were harvested, washed and resuspended in fresh medium before cell exposure in 6 µg/ml of KM for 1 h at room temperature. Total RNA of *M. tuberculosis* MT433 and MT164 were isolated using TRIzol reagent, purified by PureLink® RNA Mini kit (Ambion, Texas, USA) and analyzed using 0.8% agarose gel electrophoresis. The result showed that a total rRNA genes including 23S rRNA, 16S rRNA and 5S rRNA genes were seen in agarose gel under UV illumination (Fig. 4.8). The concentration of RNA was determined by measuring the absorbance at wavelength 260 nm using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, USA). The concentration of each total RNA was approximately 20 ng/µl. Finally, total RNA was treated with DNaseI for DNA removal before examination of DNA contamination by PCR amplification of *sigA* encoding RNA polymerase sigma factor.

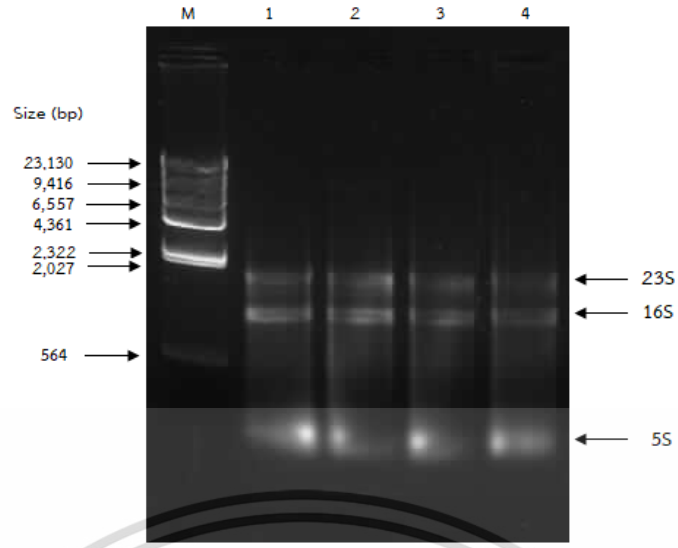


Figure 4.8 RNA of *M. tuberculosis* MT433 and MT164 strains

Lane M : 100 bp marker

Lane 1 : RNA of *M. tuberculosis* MT433

Lane 2 : RNA of *M. tuberculosis* MT433 with KM exposure

Lane 3 : RNA of *M. tuberculosis* MT164

Lane 4 : RNA of *M. tuberculosis* MT164 with KM exposure

4.3.2 Analysis of gene expression level by real-time quantitative reverse transcription PCR method (Real-time qRT-PCR)

The synthesis of cDNA was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). Primers for cDNA synthesis (RT-primer) of sixteen efflux pump genes or hypothetical transmembrane genes, *eis* and *whiB7* were designed at the specific region of each gene. The obtained cDNA was used to study the expression level of sixteen efflux pump genes or hypothetical transmembrane genes, *eis* and *whiB7* in *M. tuberculosis* MT433 and MT164 using quantitative PCR method. The expression level of genes in KM-exposed cells was compared with unexposed cells (positive control). The gene expression level of each gene under KM exposure was shown in the differential expression fold change ($2^{-\Delta\Delta Cq}$) compared with that under normal condition and with the gene expression level of *sigA* (Table 4.5 and Fig. 4.9).

The result showed that Rv2416c (*eis*) and Rv3197A (*whiB7*) gave significantly very high level of gene expression in KM-exposed *M. tuberculosis* MT433 compared to unexposed cells (Table 4.5 and Fig. 4.9). Especially, the level of gene

expression of Rv2416c (*eis*) in KM-exposed *M. tuberculosis* MT433 was 2,200 folds higher than that in KM-exposed *M. tuberculosis* MT164 (Table 4.5 and Fig. 4.9). Besides Rv2416c (*eis*) and Rv3197 (*whiB7*), seven genes comprising Rv1146, Rv1258c, Rv1410c, Rv1819c, Rv1877, Rv2846c and Rv3065 showed also a higher gene expression level in KM-exposed *M. tuberculosis* MT433 than unexposed cells. Among these seven genes, only three genes containing Rv1819c, Rv1877 and Rv2846c showed an increased level of gene expression in KM-exposed *M. tuberculosis* MT433 compared to KM-exposed *M. tuberculosis* MT164, suggesting the specific upregulation of these genes in only *M. tuberculosis* MT433 under KM exposure (Table 4.5 and Fig. 4.9). The ratio of gene expression level of Rv1819c, Rv1877 and Rv2846c was 2.6, 22.8 and 1.6 folds, respectively.

In *M. tuberculosis* MT164, Rv0194, Rv0783c, Rv1145, Rv1250 and Rv2333c were upregulated under KM exposure whereas they were downregulated under KM exposure in *M. tuberculosis* MT433 (Table 4.5 and Fig. 4.9). Moreover, the lower level of gene expression in Rv1634, Rv1457c and Rv1458c was found in both KM-exposed *M. tuberculosis* strains (Table 4.5 and Fig. 4.9). In contrast, Rv1146, Rv1258c, Rv1410c, Rv1456c, Rv1819c, Rv3065 and Rv3239c showed upregulation in both KM-exposed *M. tuberculosis* strains (Table 4.5 and Fig 4.9). In this study, three efflux pump genes including Rv1819c, Rv1877 and Rv2846c, specifically and highly expressed under KM exposure in *M. tuberculosis* MT433 were selected and studied the function of these efflux pump genes related to aminoglycoside resistance.

Table 4.5 Expression fold change of genes under KM exposure in *M. tuberculosis* MT433 and MT164

Genes	The differential expression fold change ($2^{-\Delta\Delta Cq}$)	
	MT433	**MT164**
Rv0783c	0.1	24.7
Rv0194	0.9	1.0
Rv1250	0.3	1.3
Rv1258c (*tap*)	6	4×10^7
Rv1145	0.2	195
Rv1146	1.3	4.2
Rv1410c	2	3.6
Rv1456c	1.0	1.0
Rv1457c	0	0
Rv1458c	0.8	0.7
Rv1634	0.7	0.5
Rv1819c	2.6	1.9
Rv1877	22.8	1.0
Rv2333c	0.7	1.1
Rv2846c	1.6	0.6
Rv3065	1.2	2.7
Rv2416c (*eis*)	2.2×10^3	0.9
Rv3197A (*whiB7*)	2×10^7	7×10^6

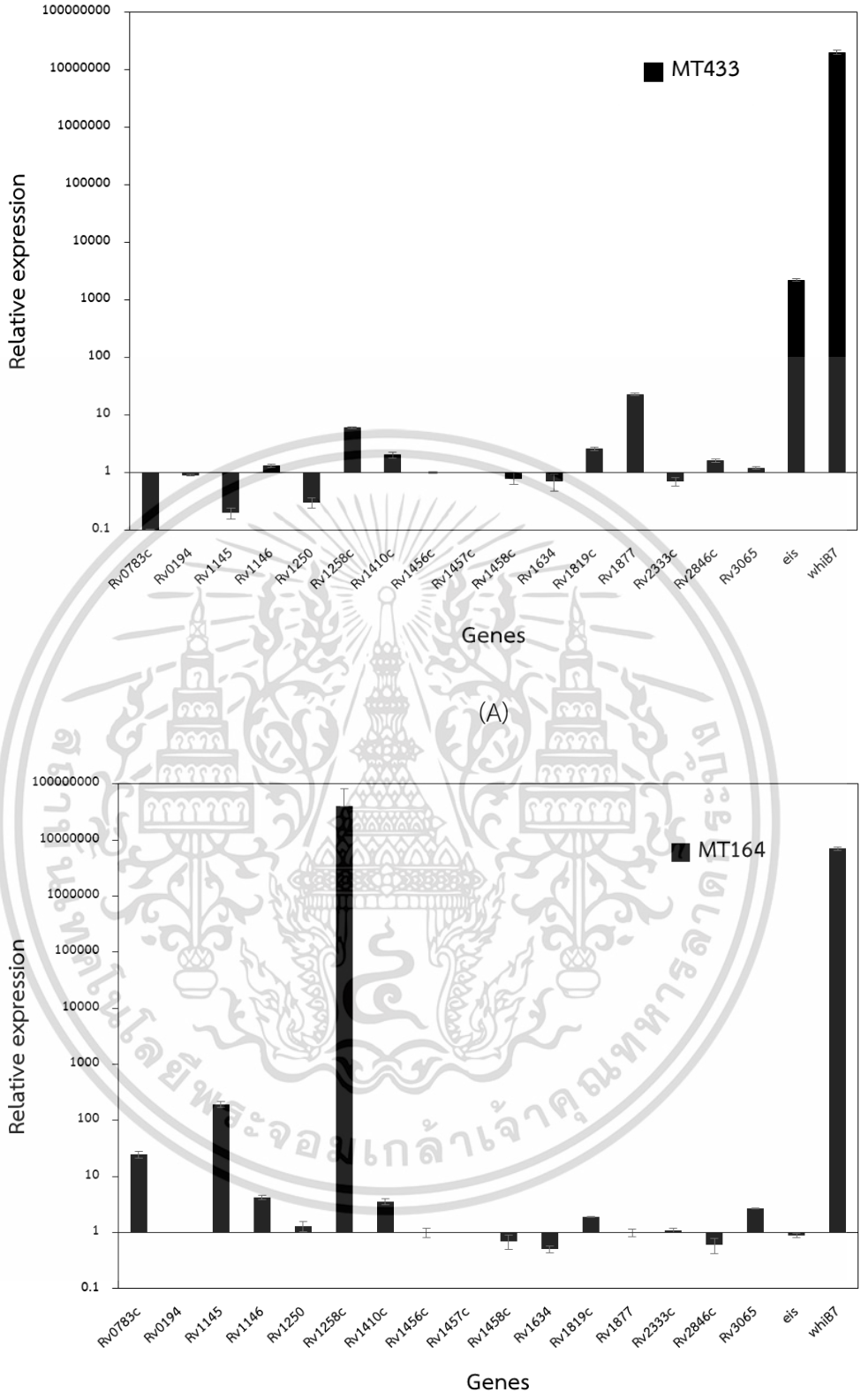


Figure 4.9 Relative expression of efflux pump genes in KM-resistant *M. tuberculosis* MT433 with KM exposed condition (A) and KM-resistant *M. tuberculosis* MT433 with KM exposed condition (B)

4.4 Construction of overexpressed *M. tuberculosis* H37Ra strains containing recombinant plasmids pSMT1 carrying Rv1819c, Rv1877 and Rv2846c

In order to determine whether high upregulated efflux pump genes, Rv1819c, Rv1877 and Rv2846c, could directly affect to the aminoglycoside resistance mechanism, recombinant plasmids pSMT1 carrying each Rv1819c, Rv1877 and Rv2846c with sense (S) and antisense (AS) directions, were constructed and transformed into *M. tuberculosis* H37Ra. Briefly, Rv1819c, Rv1877 and Rv2846c genes of KM-resistant *M. tuberculosis* MT433 were amplified by PCR and ligated with the TA cloning vector pDrive (Qiagen, Hilden, Germany). The recombinant plasmids pDrive-Rv1819c, pDrive-Rv1877 and pDrive-Rv2846c were transformed into competent cells of *E. coli* DH5α. All three genes were sequenced before sub-cloning into *E.coli/Mycobacterium* shuttle vector pSMT1 by digestion with restriction enzyme *Bam*HI. The recombinant plasmids pSMT1-Rv1819c, pSMT1-Rv1877 and pSMT1-Rv2846c were transformed into competent cells of *M. tuberculosis* H37Ra by electroporation. Transformants were selected on Middlebrook 7H10 agar containing antibiotic hygromycin. The aminoglycoside susceptibility of overexpressed *M. tuberculosis* H37Ra strains were investigated.

4.4.1 Amplification of Rv1819c, Rv1877 and Rv2846c by PCR

Primers for Rv1819c, Rv1877 and Rv2846c amplification were designed based on the published genome sequence of *M. tuberculosis* H37Rv (Accession no. NC_000962.3) and their sequences are shown in Table 3.6. The nucleotide sequence of *Bam*HI recognition site was added at position 5' end of forward and reverse primers. Rv1819c, Rv1877 and Rv2846c genes were amplified using genomic DNA of KM-resistant *M. tuberculosis* MT433 as a template. PCR reactions and conditions were described in Table 3.7. The obtained PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis. The result showed that there was only one PCR product band of each Rv1819c, Rv1877 and Rv2846c with an approximate size of 1,900, 2,000 and 1,600 bp, respectively (Fig. 4.10). These PCR product sizes were correlated with the expected sizes of 1,932, 2,076 and 1,605 bp, respectively. No PCR products of each gene were found in negative control using deionized water as a template (Fig.

4.10). All PCR products were purified using Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan) before ligation.

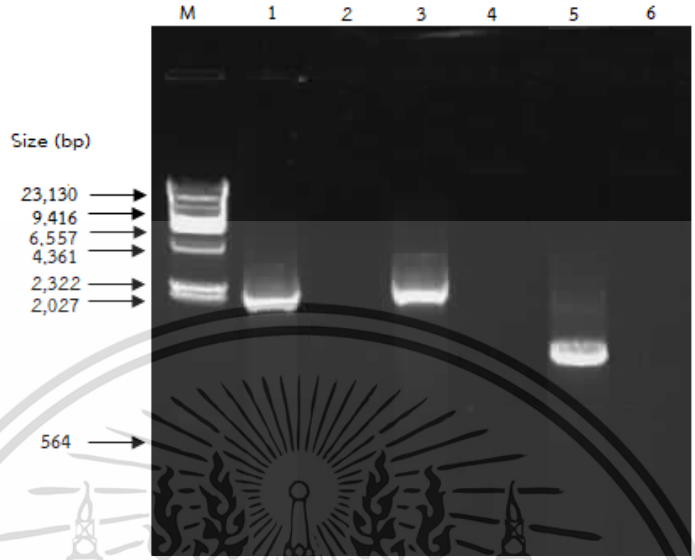


Figure 4.10 PCR products of Rv1819c, Rv1877 and Rv2846c of KM-resistant *M. tuberculosis* MT433

Lane M : λ DNA/*Hin*dIII fragments marker
Lane 1 : PCR product of Rv1819
Lane 2 : Negative PCR product of Rv1819
Lane 3 : PCR product of Rv1877
Lane 4 : Negative PCR product of Rv1877
Lane 5 : PCR product of Rv2846c
Lane 6 : Negative PCR product of Rv2846c

4.4.2 Construction of recombinant plasmids pDrive carrying each Rv1819c, Rv1877 and Rv2846c

All purified PCR products of each Rv1819c, Rv1877 and Rv2846c gene were ligated to TA cloning vector pDrive and the recombinant plasmids were transformed into competent cells *E. coli* DH5α. The transformation efficiency was about 10^6 CFU/μg DNA. Approximately 100 blue-white colonies were exhibited on LB agar containing 50 μg/ml of KM, 50 μM IPTG and 80 μg/ml of X-gal. White colonies obtained from transformation of each recombinant plasmid were selected. The colonies were cultivated in LB broth supplemented with 50 μg/ml of KM. The

recombinant plasmids were subsequently isolated by High-speed plasmid DNA mini kit (Geneaid, Taiwan) and analysed by 0.8% (w/v) agarose gel electrophoresis. The recombinant plasmids pDrive-Rv1819c, pDrive-Rv1877 and pDrive-Rv2846c are shown in Fig 4.11.

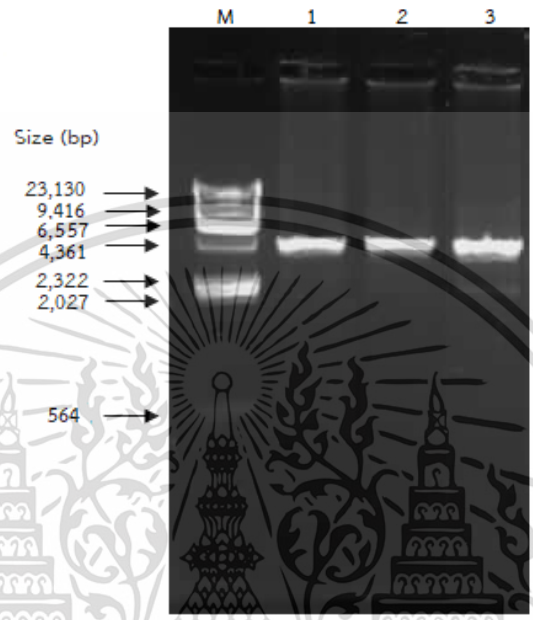


Figure 4.11 Recombinant plasmids pDrive carrying Rv1819c, Rv1877 and Rv2846c
Lane M : λ DNA/*Hin*dIII fragments marker
Lane 1 : Recombinant plasmid pDrive-Rv1819c
Lane 2 : Recombinant plasmid pDrive-Rv1877
Lane 3 : Recombinant plasmid pDrive-Rv2846c

Recombinant plasmids pDrive-Rv1819c, Rv1877 and Rv2846c were digested by restriction endonuclease *Eco*RI for confirmation of the existence of each PCR product. The digested reactions were analysed by 0.8% (w/v) agarose gel electrophoresis. The result showed that all products after *Eco*RI digestion contained two bands of pDrive with a size of 3,850 bp and each PCR product of Rv1819c, Rv1877 and Rv2846c genes with approximate sizes of 1,900, 2,000 and 1,600 bp, respectively (Fig 4.12). All sizes of *Eco*RI-digested recombinant plasmids were related to predicted sizes of TA-cloning vector pDrive and each PCR product, indicating the correct transformants containing the expected recombinant plasmids. The recombinant plasmids pDrive-Rv1819c, Rv1877 and Rv2846c isolated from selected transformants were confirmed by nucleotide sequencing using specific primers. Their

nucleotide sequences were aligned to genome of *M. tuberculosis* H37Rv. It was found that the nucleotide sequences of all genes investigated showed 100% homology to those of *M. tuberculosis* H37Rv (Appendix E). The *Bam*HI recognition site at start and stop codons of Rv1819c, Rv1877 and Rv2846c was found (Fig. 4.13).

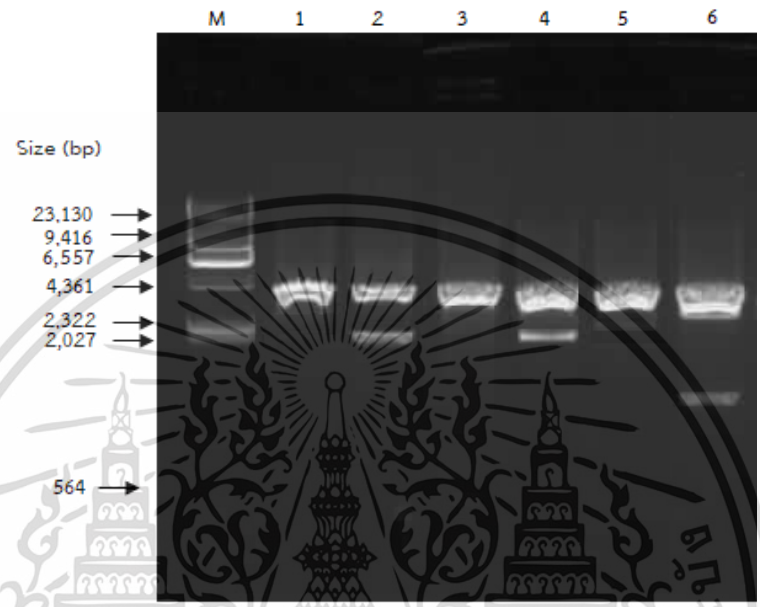


Figure 4.12 Analysis of recombinant plasmid DNA digestion by *Eco*RI using 0.8% (w/v) agarose gel electrophoresis

Lane M : λ DNA/*Hin*dIII fragments marker

Lane 1 : Recombinant plasmid DNA pDrive-Rv1819c

Lane 2 : Recombinant plasmid DNA pDrive-Rv1819c digested with *Eco*RI

Lane 3 : Recombinant plasmid DNA pDrive-R1877

Lane 4 : Recombinant plasmid DNA pDrive-1877 digested with *Eco*RI

Lane 5 : Recombinant plasmid DNA pDrive-Rv2846c

Lane 6 : Recombinant plasmid DNA pDrive-Rv2846c digested with *Eco*RI

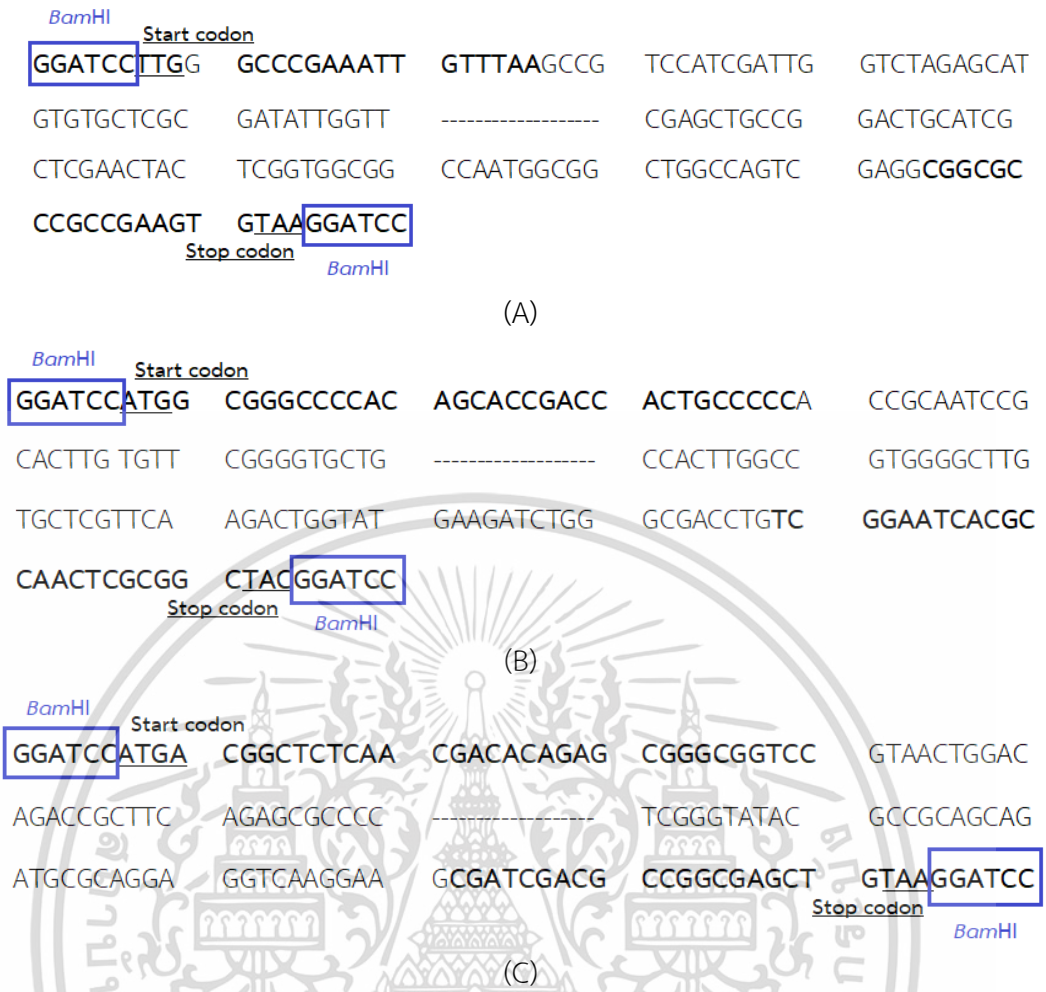


Figure 4.13 Nucleotide sequences of Rv1819c (A), Rv1877 (B) and Rv2846c (C) show *Bam*HI recognition site before start codon and after stop codon

4.4.3 Construction of recombinant plasmids pSMT1 carrying Rv1819c, Rv187 and Rv2846c

The strategy of recombinant plasmid pSMT1 containing Rv1819c, Rv1877 and R2846c construction is summarized in Fig. 4.14. The *E.coli/ Mycobacterium* shuttle vector pSMT1 was isolated and digested by *Bam*HI with before analysis woth 0.8% (w/v) agarose gel electrophoresis.

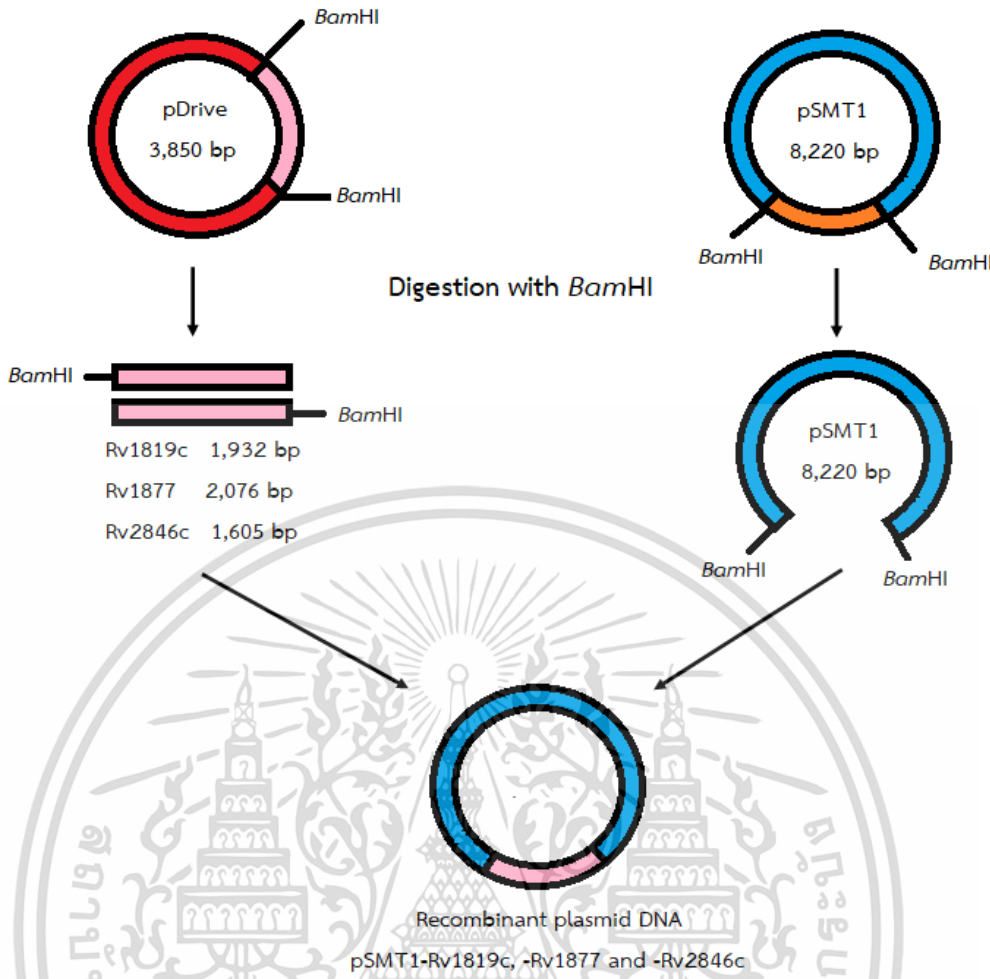


Figure 4.14 Strategy of recombinant plasmid construction of pSMT1 containing Rv1819c, Rv1877 and R2846c

The result showed that the *Bam*HI-digested pSMT1 contained two bands of a 6,177-bp linearized fragment and 2,043-bp of *luxAB* fragment (Fig 4.15). The 6,177-bp linearized fragment of *Bam*HI-digested pSMT1 was purified by Gel/PCR DNA Fragments Extraction kit and dephosphorylated by a calf intestine alkaline phosphatase (CIAP).

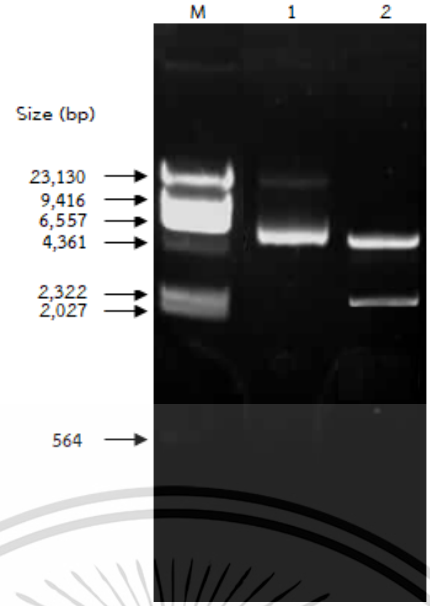


Figure 4.15 Analysis of pSMT1 digestion by restriction enzyme *Bam*HI

Lane M : λ DNA/*Hin*dIII fragments marker

Lane 1 : Plasmid pSMT1

Lane 2 : Plasmid pSMT1 digested with *Bam*HI

Three recombinant plasmids pDrive-Rv1819c, pDrive-Rv1877 and pDrive-Rv2846c were digested by *Bam*HI with components and conditions described in Table 3.10. The Rv1819c, Rv1877 and R2846c fragments obtained from *Bam*HI digestion of recombinant plasmids were purified using Gel/PCR DNA Fragments Extraction kit. All purified DNA fragments were analysed by 0.8% (w/v) agarose gel electrophoresis. The result showed that the purified dephosphorylated *Bam*HI-digested pSMT1 and the purified Rv1819c, Rv1877 and Rv2846c fragments showed each one band with an expected DNA fragment size (Fig. 4.16).

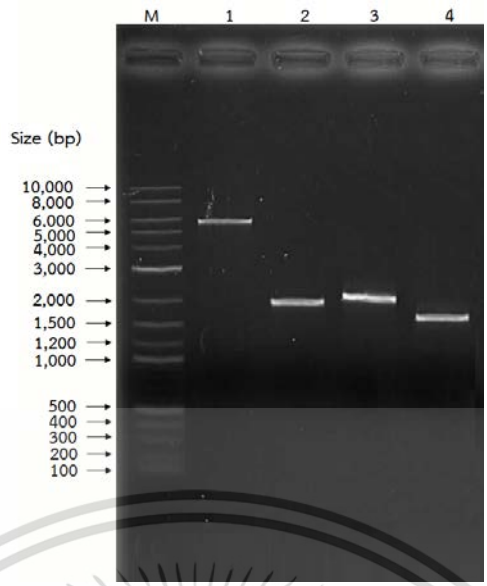


Figure 4.16 Analysis of plasmid pSMT1 and DNA fragments after dephosphorylation and purification

Lane M : 2-log DNA marker

Lane 1 : pSMT1 after dephosphorylation and purification

Lane 2 : Rv1819c after purification

Lane 3 : Rv1877 after purification

Lane 4 : Rv2846c after purification

Each purified Rv1819c, Rv1877 and Rv2846c fragment was ligated to purified dephosphorylated *Bam*HI-digested pSMT1. The recombinant plasmids pSMT1-Rv1819c, pSMT1-Rv1877 and pSMT1-Rv2846c were transformed into competent cell of *E. coli* DH5α with a transformation efficiency of 10^5 CFU/μg. Transformants were selected on LB agar supplemented with 50 μg/ml of hygromycin. A total of approximate 100 colonies were found and five transformants obtained from each gene were randomly selected for sense or antisense direction analysis. The direction of Rv1819c, Rv1877 and Rv2846c fragments in the recombinant plasmid pSMT1 was analysed by restriction enzyme *Nru*I digestion. The recombinant plasmids pSMT1-Rv1819c, pSMT1-Rv1877 and pSMT1–Rv2846c of random transformants were isolated using High-Speed Plasmid DNA mini kit and analysed by 0.8% (w/v) agarose gel electrophoresis. Figure 4.17 shows the similar and different sizes of DNA bands of isolated recombinant plasmid DNA.

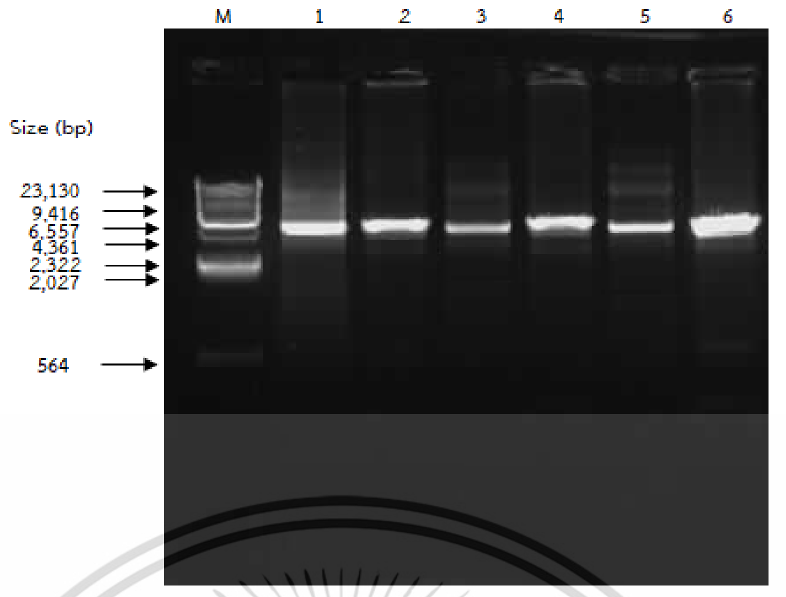


Figure 4.17 Recombinant plasmid DNA pSMT1 containing Rv1819c, Rv1877 and Rv2846c

Lane M : λ DNA/*Hin*dIII fragments marker

Lane 1 : pSMT1-Rv1819c 1

Lane 2 : pSMT1-Rv1819c 2

Lane 3 : pSMT1-Rv1877 1

Lane 4 : pSMT1-Rv1877 2

Lane 5 : pSMT1-Rv2846c 1

Lane 6 : pSMT1-Rv2846c 2

The direction analysis of each gene in recombinant plasmids pSMT1-Rv1819c, pSMT1-Rv1877 and pSMT1-Rv2846c was performed by restriction enzyme *Nru*I digestion and the digested products were analysed by 0.8% (w/v) agarose gel electrophoresis. The result showed that pSMT1-Rv1819c-1 contained a sense (S) direction of Rv1819c from the appearance of three bands with a size of approximately 4,862, 1,775 and 282 bp. pSMT1-Rv1819c-2 contained an antisense (AS) direction of Rv1819c from the appearance of three bands with a size of approximately 3,142, 2,002 and 1,775 bp (Fig. 4.18). In Rv1877, pSMT1-Rv1877-1 contained a sense (S) direction of Rv1877 from the appearance of three bands with a size of approximately 4,009, 1,775 and 1,279 bp whereas pSMT1-Rv1877-2 contained an antisense (AS) direction of Rv1877 from the appearance of three bands with a size of approximately 4,139, 1,775 and 1,149 bp (Fig. 4.18).

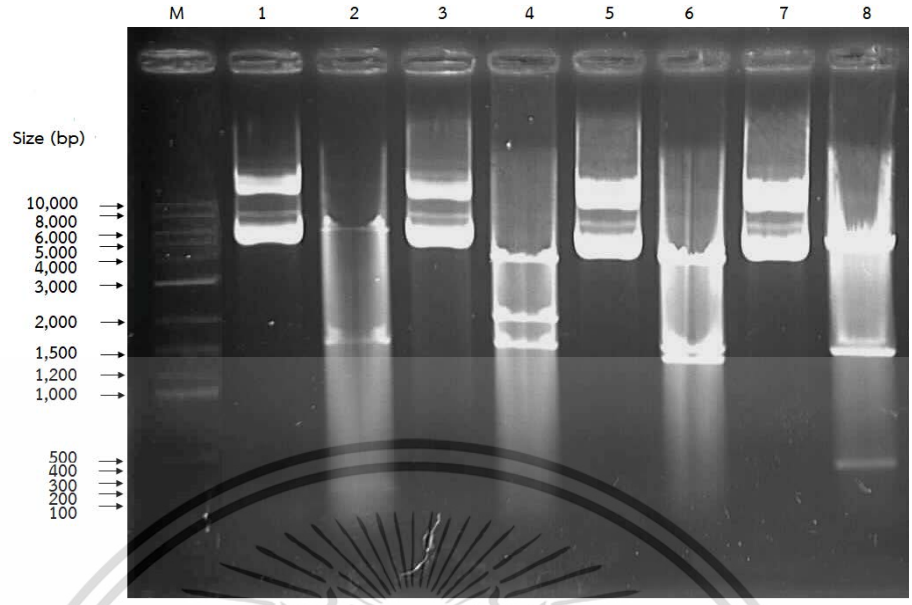


Figure 4.18 Direction analysis of recombinant plasmids pSMT1-Rv1819c and pSMT1-Rv1877 with *Nru*I digestion

Lane M : 2-log marker

Lane 1 : pSMT1-Rv1819c 1

Lane 2 : pSMT1-Rv1819c 1 digested with *Nru*I (S)

Lane 3 : pSMT1-Rv1819c 2

Lane 4 : pSMT1-Rv1819c 2 digested with *Nru*I (AS)

Lane 5 : pSMT1-Rv1877 1

Lane 6 : pSMT1-Rv1877 1 digested with *Nru*I (S)

Lane 7 : pSMT1-Rv1877 2

Lane 8 : pSMT1-Rv1877 2 digested with *Nru*I (AS)

In Rv2846c, pSMT1-Rv2846c-1 contained a sense (S) direction of Rv2846c from the appearance of three bands with a size of approximately 3,587, 1,775 and 1,230 bp whereas pSMT1-Rv2846c-2 contained an antisense (AS) direction of Rv2846c from the appearance of three bands with a size of approximately 4,090, 1,775 and 727 bp (Fig. 4.19). Finally, all recombinant plasmids with sense and antisense directions were transformed into *M. tuberculosis* H37Ra.

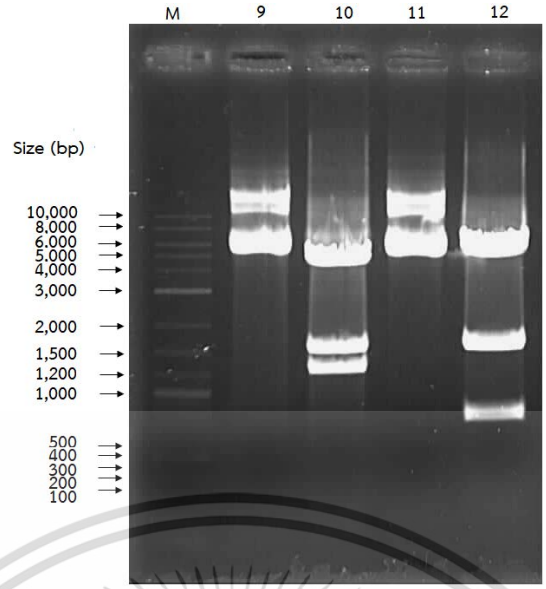


Figure 4.19 Direction analysis of recombinant plasmids pSMT1-Rv2846c with *Nru*I digestion

Lane M : 2-log marker

Lane 1 : pSMT1-Rv2846c 1

Lane 2 : pSMT1-Rv2846c 1 digested with *Nru*I (S)

Lane 3 : pSMT1-Rv2846c 2

Lane 4 : pSMT1-Rv2846c 2 digested with *Nru*I (AS)

4.5 Drug susceptibility testing and determination of minimal inhibitory concentration (MICs) of Rv1819c, Rv1877 and Rv2846c overexpressed *M. tuberculosis* H37Ra

Approximately 1 µg of recombinant plasmids pSMT1-Rv1819c-1 (S), pSMT1-Rv1819c-2 (AS), pSMT1-Rv1877-1 (S), pSMT1-Rv1877-2 (AS), pSMT1-Rv2846c-1 (S) and pSMT1-Rv2846c-2 (AS) were transformed into competent cells of *M. tuberculosis* H37Ra using electroporation with a transformation efficiency about 10^6 CFU/ml. *M. tuberculosis* H37Ra is known to be sensitive to aminoglycosides. The transformants were screened on Middlebrook 7H10 agar supplemented with 50 µg/ml of hygromycin. Three and two transformants containing each recombinant plasmid in sense (S) and antisense (AS) directions, respectively, were randomly selected. In this study, KM and AMK susceptibility test was performed using agar dilution method on Middlebrook 7H10 (Becton Dickinson, New Jersey, USA). The

different concentrations of KM and AMK (0, 2, 4, 8, 16, 32 and 64 µg/ml) were used in this study. *M. tuberculosis* H37Ra and *M. tuberculosis* H37Ra containing plasmid pSMT1 without any genes were used as control strains. The result showed that MICs to KM and AMK of all overexpressed *M. tuberculosis* H37Ra strains carrying Rv1819c, Rv1877 and Rv2846c did not show significant differences with control strains (Table 4.6), indicating that none of all strains could confer either AMK and KM resistance.

Table 4.6 MICs of amikacin and kanamycin of overexpressed *M. tuberculosis* H37Ra strains

Strains	MIC (µg/ml)	
	Amikacin	Kanamycin
H37Ra	4	4
pSMT1	4	4
pSMT1-Rv1819c 1 (S)	4	4
pSMT1-Rv1819c 2 (AS)	4	4
pSMT1-Rv1877 1 (S)	4	4
pSMT1-Rv1877 2 (AS)	4	4
pSMT1-Rv2846c 1 (S)	4	4
pSMT1-Rv2846c 2 (AS)	4	4

CHAPTER 5
Discussions

5.1 Genetic characterization of amikacin (AMK), kanamycin (KM) and capreomycin (CAP) resistance in KM-resistant *M. tuberculosis* clinical strains isolated in Thailand

In this study, the genetic characterization of genes associated with aminoglycoside resistance was investigated in twenty-nine KM-resistant *M. tuberculosis* clinical strains isolated in Thailand compared with that in twenty-seven KM-susceptible strains. The majority of mutation in *rrs* is A1401G mutation which was found in a total of twenty-one from twenty-nine KM-resistant clinical strains (72.4% of KM-resistant strains) (Table 4.1). No *rrs* mutation was found in twenty-seven KM-susceptible strains (Table 4.2). Almost *M. tuberculosis* strains with A1401G *rrs* mutation showed a high-level resistance to both AMK and KM (64 µg/ml) (Table 4.1). This result is correlated with the previous reports indicating that A1401G mutation in *rrs* is a common point mutation conferring aminoglycoside resistance mechanism and is a strong marker for selection of AMK-resistant and KM-resistant *M. tuberculosis* strains in a clinical test (Jugheli *et al*., 2009 ; Villelas *et al*., 2008). It has been reported that the *rrs* mutation at position 1401 by nucleotide substitution from A to G (A1401G) in *M. tuberculosis* (corresponding to the position 1408 in *rrs* of *E. coli* (Suzuki, 1998) was mostly found in KM-resistant *M. tuberculosis* strains (approximately 30-90% of resistant strains) that conferred a high-level resistance to AMK and KM (Ajbani *et al*., 2011 ; Evans *et al*., 2010 ; Perdigáo *et al*., 2010 ; Yuan *et al*., 2012). In addition, the other *rrs* mutations including nucleotide substitution from C to T at position 1402 (C1402T) and nucleotide substitution from G to T at position 1484 (G1484T) have also been reported (Kiet *et al*., 2010 ; Brossier *et al*., 2010 ; Feuerriegel *et al*., 2009 ; Suzuki *et al*., 1998 ; Sirgel *et al*., 2012). The C1402T and G1484T *rrs* mutations were a poor maker because these mutations could be found in KM-susceptible strains (Georghiou *et al*., 2012).

Our result also showed that A1401G *rrs* mutation was a cause of the cross-resistance to CAP with a broad MICs ranging from 4 to >64 µg/ml (Table 4.1). This result was consistent with a previous report (Jugheli *et al*., 2009). About 27.6% of KM-resistant strains (or eight from twenty-nine KM-resistant *M. tuberculosis* clinical strains) without A1401G mutation in *rrs* showed a high-level resistance to KM (>64 µg/ml) and various MICs values for AMK (8 and >64 µg/ml) (Table 4.1). From this study, the presence of A1401G mutation was a main cause of aminoglycoside resistance mechanism in KM-resistant *M. tuberculosis* clinical strains isolated in Thailand.

Besides *rrs* gene, other genes associated aminoglycoside resistance mechanism such as *eis* including promoter, *tap*, *whiB7* and *tlyA*, were investigated in both twenty-nine KM-resistant and twenty-seven KM-susceptible *M. tuberculosis* clinical strains. Eis is an aminoglycoside acetyltransferase enzyme that can transfer acetyl group of acetyl-coenzyme A to amino group of aminoglycoside drugs at the position 2'-, 3- or 6', resulting in an inactivation of these drugs (Chen *et al*., 2011). In this study, five of twenty-nine KM-resistant strains exhibited mutation in promoter region of *eis* (Table 4.1). The nucleotide substitution from C to T at position -14 (C-14T) of *eis* mutation was found in four KM-resistant strains without *rrs* mutation whereas the nucleotide substitution from G to T at position -37 (G-37T) was found in only one KM-resistant strain without *rrs* mutation (Table 4.1). Interestingly, all KM-resistant strains containing C-14T and G-37T mutations had a high-level resistance to KM and CAP (>64 µg/ml) (Table 4.1). This result is contrasted with the other reports which showed a low-level resistance to KM (4 µg/ml) in KM-resistant *M. tuberculosis* harbouring C-14T and G-37T mutations (Zaunbrecher *et al*., 2009). Above the previously-mentioned *eis* promoter mutations, the other positions of mutation in *eis* promoter region have also been reported such as G-6T, G-10A and C-12T *eis* promoter mutations (Zaunbrecher *et al*., 2009 ; Engström *et al*., 2011 ; Gikalo *et al*., 2012). However, the high-level resistance to KM were found in KM-resistant *M. tuberculosis* clinical strains without *rrs* mutation and *eis* promoter region mutation, suggesting the availability of the additional or unknown mechanisms associated with KM resistance.

Tap is a putative multidrug efflux pump protein that is encoded from *tap* gene in *Mycobacterium fortuitum* (Aínsa *et al*., 1998). Rv1258c of *M. tuberculosis* is a homologous gene with *tap* of *M. fortuitum* (Aínsa *et al*., 1998). The overexpression of *M. fortuitum* tap conferred a low-level to tetracycline (8 to 16 µg/ml) and streptomycin (2 to 16 µg/ml) resistance when expressed in *M. smegmatis* mc^2 155 (Aínsa *et al*., 1998 ; Siddiqi, 2004). In this study, the insertion of cytosine at position 581 of *tap* (Ins581C) was observed in twenty-one of twenty-nine KM-resistant *M. tuberculosis* clinical strains and five of twenty-seven KM-susceptible *M. tuberculosis* strains (Table 4.3). This Ins581C mutation makes a reduction of Tap amino acids (from 231 to 419 amino acids), resulting in an incompletion of Tap protein. By a consideration of our finding, the Ins581C mutation was not related to aminoglycoside resistance because this mutation was found in both KM-resistant and KM-susceptible *M. tuberculosis* strains. In the recent report, the Ins581C mutation was a common mutation that found in *M. tuberculosis* Beijing strains isolated from various countries including Russia, South Africa, the United Kingdom and Spain (Köser *et al*., 2013 ; Villelas *et al*., 2013).

The transcription regulator WhiB7 of *M. tuberculosis* is encoded by Rv3917A (*whiB7*) of *M. tuberculosis* that controlled the regulatory system associated with second-line drugs resistance (Burian et al., 2012 and Reeves *et al*., 2013 ; Morris *et al*., 2015). The mutations in the 5' untranslated region (UTR) of *whiB7* conferred a low-level resistance to kanamycin (10 to 20 µg/ml) and streptomycin (8 to 10 µg/ml) due to an increase of expression level of *eis* (23-fold) and *tap* (145-fold) (Reeves *et al*., 2013). However, no mutations in UTR of *whiB7* were found in both studied KM-resistant and KM-susceptible *M. tuberculosis* strains (Table 4.1), suggesting that UTR mutation of *whiB7* was not related to aminoglycoside resistance in *M. tuberculosis* isolated in Thailand.

In the previous studies, the mutation of *tlyA* (encoding 2'-O-methyltransferase) conferred capreomycin resistance and was related to cross-resistance of KM in *M. tuberculosis* (Engström *et al*., 2011 ; Johansen *et al*., 2006 ; Maus *et al*., 2005). In this study, A33G, Ins49GC and T539G mutations in *tlyA* were investigated in tested *M. tuberculosis* strains (Table 4.1). The A33G mutation in *tlyA* has been reported as a polymorphism in *M. tuberculosis* Beijing strains (Maus *et al*., 2005). The other

mutations in *tlyA* were related to high-level resistance to CAP (64 µg/ml) in KM-resistant strains without *rrs* mutation. In addition, T539G mutation was found in *M. tuberculosis* strains isolated in Korea but it showed in a low percentage of all resistant strains (approximately 3.5%) (Jnawali *et al*., 2013).

5.2 Analysis of KM-resistant *M. tuberculosis* MT433 by whole genome sequencing (WGS)

In this study, whole genome sequences of KM-resistant *M. tuberculosis* MT433 (DS no. 24433) were analysed by WGS. *M. tuberculosis* MT433 is an interesting KM-resistant isolate for WGS study because it has no mutations of *rrs*, *eis* including promoter, *tap*, *whiB7* and *tlyA*. In the annotated result of WGS, 342 nonsynonymous, 218 synonymous and 273 intergenic were shown in *M. tuberculosis* MT433 when compared with genome of *M. tuberculosis* H37Rv (Appendix D). From WGS data of *M. tuberculosis* MT433, the nucleotide sequences of sixteen efflux pump and hypothetical transmembrane genes were focused. The nucleotide sequence of Rv1250 showed nucleotide substitution from cytosine to thymine at the position 1506 (C1506T) but this mutation did not cause an amino acid change (Table 4.4). In addition, the nucleotide substitution from thymine to cytosine at the position 221 (T221C) of Rv0194 was a mutation found by WGS analysis (Table 4.4).

Four groups of efflux pump or hypothetical transmembrane genes were investigated in this study. The Rv0783c, Rv1250, Rv1258c, Rv1410c, Rv1634, Rv1877, Rv2333c and Rv2846c were categorized in Major Facilitator Superfamily (MFS) which uses a bacterial proton motive force for driving transporters (Mazukiewicz *et al*., 2005). In the previous reports, these MFS genes had been shown to relate with multidrug resistance efflux pump in *M. tuberculosis*, especially a second-line antituberculosis drugs (De Rossi *et al*., 2002 ; Pang *et al*., 2013 ; Mazukiewicz *et al*., 2005). The Rv1456c, Rv1457c, Rv1458c and Rv1819c were categorized in ATP-Binding Cassette (ABC) family that conferred resistance of multidrug such as streptomycin, ampicillin, tetracycline and aminoglycoside (Hao *et al*., 2011 ; Domenech *et al*., 2009 ; Danilchanka *et al*., 2008). The Rv1145 and Rv1146 were categorized in Resistance Nodulation Division (RND) family that were a homologous with drug resistance efflux

pump *mmpL* gene in *M. smegmatis* (Li *et al*., 2004). The Rv3065 was categorized in Small Multidrug Resistance (SMR) that showed a low-level resistance to KM in *M. smegmatis* (Li *et al*., 2004). In conclusion, nucleotide sequences of these genes could not correlate with KM resistance in *M. tuberculosis* MT433.

5.3 Investigation of expression level of putative efflux pump genes using real-time qRT-PCR

The expression level of sixteen efflux pump or hypothetical transmembrane genes, *eis* and *whiB7* genes of KM-resistant *M. tuberculosis* MT433 were analysed using real-time qRT-PCR. The gene expression of KM-resistant *M. tuberculosis* MT433 and MT164 (strain without A1401G) were determined under KM-exposed and KM-unexposed conditions. The result showed that the very high level of gene expression of Rv3197A (*whiB7*) was found in both *M. tuberculosis* MT433 and MT164 strains under KM exposure (Table 4.5), indicating that high level of Rv3197A (*whiB7*) expression is not involved in aminoglycoside resistance mechanism. Interestingly, the high level of gene expression of Rv2416c (*eis*) (2,444 expression fold change) was found in only *M. tuberculosis* MT433 under KM exposure (Table 4.5), suggesting the possibility of the *eis* gene expression in aminoglycoside resistance. Eis is produced during tuberculosis infection in human and it is directly correlated with survival effector of macrophage within human and can be found in anaerobic bacilli including mycobacteria (Black *et al*., 2014 ; Sarathy *et al*., 2012). In addition, the point mutation in promoter region of *eis* correlates with a low-level to KM in *M. tuberculosis* (with MICs of 5 to 40 µg/ml) (Zaunbrecher *et al*., 2009). *M. tuberculosis* carrying *eis* promoter mutation increased 20-180 folds of *eis*-transcribed protein (Zaunbrecher *et al*., 2009). Interestingly, no mutations in *eis* promoter region were found in *eis* of *M. tuberculosis* MT433 but high expression level of *eis* was detected in this study, indicating that *eis* might be involved in a second aminoglycoside resistance mechanism in KM-resistant *M. tuberculosis* MT433.

Besides *whiB7* and *eis*, three genes including Rv1819c, Rv1877 and Rv2846c showed a high expression level in *M. tuberculosis* MT433 under KM exposure (Table 4.4). The ABC transporter gene Rv1819c of *M. tuberculosis* was homologous with

bacA (encoding a putative cytoplasmic membrane transport protein) of *Brucella adortusi* (gram-negative alphaproteobacterium) (39% similarity) and was found to contribute to the maintenance of chronic tuberculosis infectious system in mice (Domenech *et al*., 2009). In addition, *bacA* deficient *M. tuberculosis* strain conferred a low-level resistance to bleomycin (Domenech *et al*., 2009). Considering of a level of Rv1819c expression, approximately 2.6 expression fold change was found in *M. tuberculosis* MT433 under KM exposure (Table 4.5). Focusing on the level of gene expression in Rv1877, approximately 22.8 expression fold change was found in *M. tuberculosis* MT433 under KM exposure compared to unexposed *M. tuberculosis* MT433 and also *M. tuberculosis* MT164 under KM exposure (Table 4.5) The Rv1877 in *M. tuberculosis* showed 58% similarity to *M. smegmatis* Rv1877 and the lack of Rv1877 increased erythromycin, novobiocin, tetracycline and KM susceptibility values in *M. smegmatis* (Li *et al*., 2004). For Rv2846c, it has been reported that the deficiency of Rv2846c conferred ethidium bromide and acriflavine resistance in *M. smegmatis* (Li *et al*., 2004). In our result, the Rv1877 and Rv2846c were upregulated in *M. tuberculosis* MT433 under KM exposure but were downregulated in *M. tuberculosis* MT164 (Table 4.5), indicating that Rv1877 and Rv2846c are probably related to aminoglycoside resistance in KM-resistant *M. tuberculosis* MT433.

5.4 Determination of overexpressed genes on amikacin and kanamycin susceptibility in *M. tuberculosis* H37Ra

The Rv1819c, Rv1877 and Rv2846c were highly-expressed in *M. tuberculosis* MT433 under KM exposure condition. The overexpression of these genes was performed using shuttle vector pSMT1. The recombinant plasmid DNAs were finally transformed into *M. tuberculosis* H37Ra. The susceptibility test with various concentrations of AMK and KM was determined in overexpressed strains containing Rv1819c, Rv1877 and Rv2846c. No differences of MICs values to AMK and KM were found between *M. tuberculosis* H37Ra overexpressed strains and control (Table 4.6). The result suggested that Rv1819c, Rv1877 and Rv2846c did not directly associate with aminoglycoside resistance in KM-resistant *M. tuberculosis* MT433 strain. This result was not consistent with the previous reports because the deletion of Rv1877

increased susceptibility to erythromycin, novobiocin, tetracycline and kanamycin (Chen *et al*., 2011 ; Domenech *et al*., 2009 ; Hao *et al*., 2011 ; Li *et al*., 2004).

CHAPTER 6

Conclusions

6.1 Genetic characterization of amikacin (AMK), kanamycin (KM) and capreomycin (CAP) resistance in KM-resistant *M. tuberculosis* clinical strains isolated in Thailand

6.1.1 The majority of KM resistance mechanism in KM-resistant *M. tuberculosis* clinical strains isolated in Thailand was A1401G *rrs* mutation (accounting for 72.4% of KM-resistant strains). All KM-resistant *M. tuberculosis* clinical isolates with A1401G mutation showed a high-level resistance to KM (64 µg/ml).

6.1.2 Mutations of *eis* promoter region either at C-14T or G-37T were found in 17.2% of KM-resistant *M. tuberculosis* clinical strains isolated in Thailand.

6.1.3 The Ins581C mutation of *tap* resulting in a reduction of TAP amino acids were found in 6.9% of KM-resistant *M. tuberculosis* clinical strains isolated in Thailand.

6.1.4 The *tlyA* mutation at A33G known as polymorphism mutation was found in all KM-resistant and KM-susceptible *M. tuberculosis* clinical strains isolated in Thailand. In addition, the other *tlyA* mutations at Ins49GC and T539G were found in each one KM-resistant *M. tuberculosis* strains.

6.1.5 No mutations in all aminoglycoside resistant genes, including *rrs*, *eis* promoter region, *tap*, *whiB7* and *tlyA* were found in KM-resistant *M. tuberculosis* MT433; although it showed a high-level resistance to AMK and KM (>64 µg/ml).

6.2 Investigation of nucleotide sequences of efflux pump or hypothetical transmembrane genes using WGS

6.2.1 The whole genome of *M. tuberculosis* MT433 (DS no. 24433) contained a total of 4,409,112 bp which is composed of 833 SNIPs (342 nonsysnonymous, 218 synonymous and 273 intergenic)

6.2.2 In sixteen efflux pump or hypothetical transmembrane genes, *M. tuberculosis* MT433 contained the T221C nonsynonymous mutation in Rv0194 and the C502T synonymous mutation in Rv1250.

6.3 Investigation of expression level of efflux pump or hypothetical transmembrane genes using Real-time qRT-PCR and function analysis using AMK and KM susceptibility testing

6.3.1 In KM-resistant *M. tuberculosis* MT433, three genes from sixteen efflux pump or hypothetical transmembrane genes including Rv1819c, Rv1877 and Rv2846c showed a high-level gene expression (>2.6-fold change) under KM exposure.

6.3.2 By AMK and KM susceptibility testing in overexpressed *M. tuberculosis* H37Ra containing Rv1819c, Rv1877 and Rv2846c, these genes was not directly associated with aminoglycoside resistance in *M. tuberculosis* MT433.

6.3.3 The high-level gene expression of *eis* might be associated with KM resistance mechanism in *M. tuberculosis* MT433.

References

Aeschlimann, J.R. 2003. "The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa* and other gram-negative bateria. Insights from the society of infectious disease pharmacists." *Pharmacotherapy*. 23(7) : 916-924.

Aínsa, J.A. Blokpoel, M.C. Otal, I. Young, D.B. De Smet, K.A.L. and Martin, C. 1998. "Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*." *J. Bacteriol*. 180(22) : 5836-5843.

Ajbani, K. Rodrigues, C. Shenai, S. and Mehta, A. 2011. "Mutation detection and accurate diagnosis of extensively drug-resistant tuberculosis: report from a tertiary care center in India." *J. Clin. Microbiol*. 49(4) : 1588-1590.

Balganesh, M. Dinesh, N. Shama, S. Kuruppath, S. Nair, A.V. and Shama, U. 2012. "Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates." *Antimicrob. Agents Chemother.* 56(5) : 2643-2651.

Bau R., and Tsyba, I. 1999. "Crystal structure of amikacin." *Tetrahedron.* 55(52) : 14839-14846.

Becker, A. 1961. "A simple method for the cultivation of *Mycobacterium tuberculosis*." *Am. Rev. Respir. Dis.* 84(2) : 281-283.

Bardou, F. Raynaud, C. Ramos, C. Lanéelle, M.A. and Lanéelle, G. 1998. "Mechanism of isoniazid uptake in *Mycobacterium tuberculosis*." *Microbiology.* 144(9) : 2539-2544.

Black, P.A. Warren, R.M. Louw, G.E. Van Helden, P. Victor, T.C. and Kana, B.D. 2014. "Energy methbolism and drug efflux in *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 58(5) : 2491-2503.

Brossier, F. Veziris, N. Aubry, A. Jarlier, V. and Sougakoff, W. 2010. "Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethanbutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates." *J. Clin. Microbiol.* 48(5) : 1683-1689.

Burian, J. Ramón-García, S. Sweet, G. Gómez-Velasco, A. Av-Gay, Y. and Thompson, C.J. 2012. "The mycobacterial transcriptional regulator *whiB7* gene links redox homeostasis and instrinsic antibiotic resistance." *J. Biol. Chem.* 287(1) : 299-310.

Cade, C.E. Dlouhy, A.C. Medzihradszky, K.F. Salas-Castillo, S.P. and Ghiladi, R.A. 2010. "Isoniazid-resistance conferring mutations in *Mycobacterium tuberculosis* KatG: Catalase, peroxidase, and INH-NADH adduct formation activities." *Protein Sci.* 19(3) : 458-474.

Campbell, E.A. Korzheva, N. Mustaev, A. Murakami, K. Nair, S. Goldfarb, A. and Darst, S.A. 2001. "Structural mechanism for rifampicin inhibition of bacterial RNA polymerase." *Cell.* 104 : 901-912.

Casali, N. Nikolayevskyy, V. Balabanova, Y. Ignatyeva, O. Kontsevaya, I Harris, S.R. Bently, S.D. Parkhill, J. Nejentsev, S. Hoffner, S.E. Horstmann, R.D. Brown, T. and Drobniewski, F. 2012. "Microevolution of extensively drug-resistant tuberculosis in Russia." *Genome Res.* 22(4) : 735-45.

Chen, W. Biswas, T. Porter, V.R. Tsodikov, O.V. and Garneau-Tsodikova, S. 2011. "Unusual regioversatility of acetyltransferase Eis, a cause of drug resistance in XDR-TB." *Proc. Natl. Acad. Sci. U.S.A.* 108(24) : 9804-9808.

CLSI. 2003. "Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomyces; approved standard CLSI document M24-A." 6-12. in Gali, L. and Woods, MD. **Clinical and Laboratory Strandards Institue.** Wayne : CLSI.

Comas, I. Chakravartti, J. Small, P.M. Galagan, J. Niemann, S. Kremer, K. Ernst, J.D. and Gagneux, S. 2010. "Human T cell epitopes of *Mycoacterium tuberculosis* are evolutionarily hyperconserved." *Nat. Genet.* 42(6) : 498-503.

Comas, I. Borrell, S. Roetzer, A. Rose, G Malla, B. Kato-Maeda, M. Galagan, J. Niemann, S. and Gagneux, S. 2012. "Whole-genome sequencing of rifampicin-resistant

Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes." *Nat. Genet.* 44(1) : 106-110.

Cui, Z. Wang, J. Lu, J. Huang, X. and Hu, Z. 2011. "Association of mutation patterns in *gyrA/B* genes and ofloxacin resistance levels in *Mycobacterium tuberculosis* from east Chaina in 2009." *BMC Infect. Dis.* 11(78) : doi : 10.1186/1471-2334-11-78.

Danilchanka, O. Mailaender, C. and Niederweis, M. 2008. "Identification of a novel multidrug efflux pump of *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 52(7) : 2503-2511.

Dannenberg, A.M. Jr. 1993. "Immunopathogenesis of pulmonary tuberculosis." *Hosp. Pract.* 28(1) : 51-58.

De Rossi, E. Arrigo, P. Bellinzoni, M. Silva, P.A. Martin, C. Aínsa, J.A. Guglierame, P. and Riccardi, G. 2002. "The multidrug transporters belonging to major facilitator superfamily in *Mycobacterium tuberculosis*." *Mol. Med.* 8(11) : 714-724.

De Silva, P.E.A. Groll, A.V. Martin, A. and Polomino, J.C. 2011. "Efflux as a mechanism for drug rsistance in *Mycobacterium tubrcuosis*." *FEMS. Immunol. Med. Microbiol.* 63(1) : 1-9.

Desikan, P. 2013. "Sputum smear microscopy in tuberculosis: Is it still relevant?." *Indian J. Med. Res.* 137(3) : 442-444.

Dessen, A. Quémard, A. Blanchard, J.S. Jacobs, W.R.Jr. and Sacchettini, J.C. 1995. "Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*." *Science.* 267(5204) : 1638-1641.

Domenech, P. Kobayashi, H. LeVier, K. Walker, G.C. and Barry III, C.E. 2009. "BacA, an ABC transporter involved in maintenance of chronic murine infections with *Mycobacterium tuberculosis*." *J. Bacteriol.* 191(2) : 477-485.

Elbir, H. Abdel-Muhsin, A.M. and Babiker, A. 2008. "Short report: a one-step DNA PCR-based method for the detection of *Mycobacterium tuberculosis* complex grown on Löwenstein-Jensen Media." *Am. J. Trop. Med. Hyg.* 78(2) : 316-317.

Engström, A. Perskvist, N. Werngren, J. Hoffner, S.E. and Juréen, P. 2011. "Comparison of clinical isolates and in vitro selected mutants reveals that *tlyA* is not a sensitive genetic marker for capreomycin resistance in *Mycobacterium tuberculosis*." *J. Antimicrob. Chemother.* 66(6) : 1247-1254.

Evans, J. and Segal, H. 2010. "Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis*." *J. Antimicrob. Chemother.* 65(5) : 897-900.

Feuerriegel, S. Cox, H.S. Zarkua, N. Karimovich, H.A. Braker, K. Rüsch-Gerdes, S. and Niemann, S. 2009. "Sequence analysis of just four genes to detect extensively drug-resistant Mycobacterium tuberculosis strains in multidrug-resistant tuberculosis patients undergoing treatment." *Antimicrob. Agents Chemother.* 53(8) : 3353-3356.

Finken, M. Kirschner, P. Meier, A. Wrede, A. and Böttger, E.C. 1993. "Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot." *Mol. Microbiol.* 9(6) : 1239-1246.

Forrellad, M.A. Klepp, L.I. Gioffré, A. García, J.S.Y. Morbidoni, H.R. Santangelo, M.D.L.P. Cataldi, A.A. and Bigi, F. 2013. "Virulence factors of the *Mycobacterium tuberculosis* complex." *Virulence.* 4(1) : 3-66.

Gagneu, S. Long, C.D. Small, P.M. Van, T. Schoolnik, G.K. and Bohannan, B.J. 1998. "The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*." *Science.* 312(5782) : 1944-1946.

Gellert, M. O´Dea, M.H. Itoh, T. and Tomizawa, J. 1976. "Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase." *Proc. Natl. Acad. Sci. U.S.A.* 73(12) : 4474-4478.

Georghiou, S.B. Magana, M. Garfein, R.S. Catanzaro, D.G. Catanzaro, A. and Rodwell, T.C. 2012. "Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review." *PLoS One.* 7(3) : e33275.

Gikalo, M.B. Nosava, E.Y. Krylova, L.Y. and Moroz, A.M. 2012. "The role of *eis* mutations in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates from the Moscow region." *J. Antimicrob. Chemother.* 67(9) : 2107-2109.

Granfe, J.M. 1996. **Mycobacteria and human disease, 2nd ed.** London : Oxford University Press.

Guessogo, W.R. Mandengue, S.H. Ndemba, P.B.A. Medjo, U.O. Minye, E.E. Ahmaidi, S. and Temfemo, A. 2008. "Physical and functionl follow-up of tuberculosis patients in initial intensive phase of treatment in Cameroon using the 6-min walk test." *J. Exerc. Rehabil*. 12(4) : 333-339.

Gupta, A.K. Reddy, V.P. Lavania, M. Chaauhan, D.S. Vaankatesan, K. Sharma, V.D. Tyagi, A.K. and Katoch, V.M. 2010. "*jefA* (Rv2459), a drug efflux gene in *Mycobacterium tuberculosis* confers resistance to isoniazid & ethambutol." *Indian. J. Med. Res.* 132 : 176-88.

Hao, P. Shi-Liang, Z. Ju, L. Ya-Xin, D. Biao, H. Xu, W. Min-Tao, H. Shou-Gang, K. and Ke, W. 2011. "The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from *Mycobacterium tuberculosis* clinical isolates in China." *Folia. Microbiol. (Praha).* 56(6) : 549-553.

Hartmann, G. Honikel, K.O. Knüsel, F. and Nüesch, J. 1967. "The specific inhibition of the DNA-directed RNA synthesis by rifamycin." *Biochim. Biophys. Acta.* 145(3) : 843-844.

Hazbón, M.H. Valle, M.B.D. Guerrero, M.I. Varma-Basil, M. Filliol, I. Cavatore, M. Colangeli, R. Safi, H. Billman-Jacobe, H. Lavender, C. Fyfe, J. García-García, L. Davidow, A. Brimacombe, M. León, C.I. Porras, T. Bose, M. Chaves, F. Eisenach, K.D. Sifuentes-Osornio, J. De León, A.P. Cave, M.D. and Alland, D. 2005. "Role of embB codon 306 mutations in *Mycobacterium tuberculosis* revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance." *Antimicrob. Agents Chemother.* 49(9) : 3794-3802.

Hazbón, M.H. Brimacombe, M. Bobadilla, D.V.M. Cavatore, M. Guerrero, M.I. Varma-Basil, M. Billman, J.H. Lavender, C. Fyfe, J. García-García, L. León, C.l. Bose, M.

Chaves, F. Murray, M. Eisenach, K.D. Sifuentes-Osornio, J. Cave, M.D. Ponce, D.L.A. and Alland, D. 2006. "Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 50(8) : 2640-2649.

Helman, R.G. Russell, W.C. Jenny, A. Miller, J. and Payeur, J. 2000. "Diagnosis of tuberculosis in two snow leopards using polymerase chain reaction*." J. Vet. Diagn. Invest.* 10(1) : 89-92.

Herrmann, J.L. and Lagrange, P.H. 2005. "Dendritic cells and Mycobacterium tuberculosis: which is the Trojan horse?." *Pathol. Biol (Paris).* 53(1) : 35-40.

Heym, B. Zhang, Y. Poulet, S. Young, D. and Cole, S.T. 1993. "Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*." *J. Bacteriol.* 175(13) : 4255-4259.

Hooper, D.C. 1999. "Mechanisms of fluoroquinolone resistance." *Drug Resist. Updat.* 2(1) : 38-55.

Jnawali, H.N. Yoo, H. Ryoo, S. Lee, K.J. Kim, B.J. Koh, W.J. Kim, C.K. Kim, H.J. and Park, Y.K. 2013. "Molecular genetics of *Mycobacterium tuberculosis* resistant to aminoglycosides and cyclic peptide capreomycin antibiotics in Korea." *World J. Microbiol. Biotechnol.* 29(6) : 975-982.

Johansen, S.K. Maus, C.E. Pikaytis, B.B. and Douthwaite, S. 2006. "Capreomycin binds across the ribosomal subunit interface using *tlyA* encoded 2'-O-methylations in 16S and 23S rRNAs." *Mol. Cell.* 23(2) : 173-182.

Johanson, R. Jordaan, A.M. Pretorius, L. Englke, E. Van der Spuy, G. Kewley, C. Bosman, M. Van Helden, P.D. Warren, R. and Victor, T.C. 2006. "Ethambutol resistance testing by mutation detection." *Int. J. Tuberc. Lung Dis.* 10(1) : 68-73.

Jonmalung, J. Prammananan, T. Leechawengwongs, M. and Chaiprasert, A. 2010. "Surveillance of pyrazinamide susceptibility among multidrug-resistant *Mycobacterium tuberculosis* isolates from Siriraj hospital, Thailand." *BMC. Microbiol.* doi: 10.1186/1471-2180-10-223.

Jugheli, L. Bzekalava, N. De Rijk, P. Fissette, K. Portaels, F. and Rigouts, L. 2009. "High level of cross-resistance between kanamycin, amikacin, and capreomycin among *Mycobacterium tuberculosis* isolates from Georgia and a close relation with mutations in the *rrs* gene." *Antimicrob. Agents Chemother.* 53(12) : 5064-5068.

Julius, H. and Comroe, Jr. 1978. "Pay Dirt: the story of streptomycin." *Am. Rev. Respir. Dis.* 117(4) : 773-781.

Karlson, A.G. and Needham, G.M. 1948. "The in vitro activity of ethambutol (Dextro-2,2´-[Ethylenediimino-di-l-Butanol]) against tuberculosis bacilli and other microorganisms." *Am. Rev. Respir. Dis.* 83(6) : 905-906.

Kibbe, W.A. 2007. "OligoCalc: an online oligonuccleotide properties calculator." *Nucleic. Acid Res.* 35 : W43-46.

Kiet, V.S. Lan, N.T.N. An, D.D. Duang, N.H. Hoa, D.V. Chau, N.V. Chinh, N.T. Farrar, J. and Caws, M. 2010. "Evaluation of the MTBDRsl test for detection of second-line drug resistance in *Mycobacterium tuberculosis*." *J. Clin. Microbiol.* 48(8): 2934-3356.

Klastersky, J. Hengens, C. and Meunier-Carpentier, F. 1976. "Comparative effectiveness of combinations of amikacin with penicilin G and amikacin with carbenicillin in gram-negative septicemia: double-blind clinical trial." *J. Infect. Dis.* 134 : S433-S440.

Kobboldt, D.C. Zhang, Q. Larson, D.E. Shen, D. McLellan, M.D. Lin, L. Miller, C.A. Mardis, E.R. Ding, L. Wilson, R.K. 2012. "VarScan 2: somatic mutation and copy number alteration discovery in cancer by exon sequencing." *Genome Res.* 22(3) : 568-576.

Kwan, C.K. and Ernst, J.D. 2011. "HIV and tuberculosis: a deadly human syndemic." *Clin. Microbiol. Rev.* 24(2) : 351-376.

Köser, C.U. Bryant, J.M. Parkhill, J. and Peacook, S.J. 2013. "Consequences of *whiB7* (Rv3194A) mutations in Beijing genotype isolates of the *Mycobacterium tuberculosis* complex." *Antimicrob. Agents Chemother.* 57(7) : 3461.

Leite, C.Q. De Souza, C.W. and Leite, S.R. 1998. "Identification of mycobacteria by thin layer chromatographic analysis of mycolic acids and conventional biochemical method: four years of experience." *Mem. Inst. Oswaldo Cruz.* 93(6) : 801-805.

Langmead, B. Trapnell, C. Pop, M. and Salzberg, S.L. 2009. "Ultrafast and memory-efficient alignment of short DNA sequences to the human genome." *Genome Biol.* 10(3) : R25.

Li, X.Z. Zhang, L. and Nikaido, H. 2004. "Efflux pump-mediated intrinsic drug resistance in *Mycobactrium smegmatis*." *Antimicrob. Agents Chemother.* 48(7) : 2415-2423.

Li, H. Handsaker, B. Wysoker, A. Fennell, T. Ruan, J. Homer, N. Marth, G. Abecasis, G. Durbin, R. and 1000 Genome Project Data Processing Subgroup. 2009. "The sequence alignmeent/map format and SAMtools." *Bioinformatics.* 25(16) : 2078-2079.

Li, G. Zhang, J. Guo, Q. Jiang, Y. Wei, J. Zhao, L.I. Zhao, X. Lu, J. and Wan, K. 2015. "Study of efflux pump gene expression in rifampicin-monoresistant *Mycobacterium tuberculosis* clinical isolates." *J. Antibiot.* 68(7) : 431-435.

Maniatis, T. Fritsch, E.F. and Sambrook, J. 1982. **Molecular cloing – a laboratory manual.** New York : Plainview.

Mantoux, C. 1910. "L´intradermo-reaction à la tuberculine et son interpretation clinique." *Presse Med.* 18 : 10-13.

Maus, C.E. Plikaytis, B.B. and Shinnick, T.M. 2005. "Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 49(8) : 3192-3197.

Maus, C.E. Plikaytis, B.B. and Shinnick, T.M. 2005. "Mutations of tlyA confers capreomycin resistance in *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 49(2) : 571-577.

Mazurkiewicz, P. Poelarends, G.J. Driessen, A.J. and Konings, W.N. 2004. "Facilitated drug influx by an energy uncoupled secondary multidrug transporter." *J. Biol. Chem.* 279(1) : 103-108.

Mboowa, G. Namaganda, C. and Ssengooba, W. 2014. "Rifampicin resistance mutations in the 81 bp RRDR of *rpoB* gene in *Mycobacterium tuberculosis* clinical isolates using Xpert®MTB/RIF in Kampala, Uganda: a retrospective study." *BMC Infect. Dis.* 14 : 481.

Michel, A. Ventor, L. Espie, I.W. and Coetzee, M.L. 2003. "*Mycobacterium tuberculosis* infections in eight species at the National Zoological Gardens of South Africa between 1991-2001: an anthropozoonosis?." *J. Zoo Wildl. Med.* 34(4) : 364-370.

Mingeot-Leclercq, M.P. Glupczynski, Y. and Tulkens, P.M. 1999. "Aminoglycosides: activity and resistance." *Antimicrob. Agents Chemother.* 43(4) : 727-737.

Morris, R.P. Nguyen, L. Gatfield, J. Visconti, K. Nguyen, K. Schnappinger, D. Ehrt, S. Liu, Y. Heifets, L. Pieters, J. Schoolnik, G. Thompson, C.J. 2005. "Ancestral antibiotic resistance in *Mycobacterium tuberculosis*." *Proc. Natl. Acad. Sci. U.S.A.* 102(34) : 12200-12205.

Murakami, S. Nakashima, R. Yamashita, E. and Yamaguchi, A. 2002. "Crystal structure of bacterial multidrug efflux transporter AcrB." *Nature.* 419 : 587-593.

Nair, J. Rouse, D.A. Bai, G.H. and Morris, S.L. 1993. "The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*." *Mol. Microbiol.* 10(3) : 521-527.

Nishino, K. Nikaido, E. and Yamaguchi, A. 2009. "Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*." *Biochim. Biophys. Acta.* 1794(5) : 834-843.

Pang, Y. Lu, J. Wang, Y. Song, Y. Wang, S. and Zhao, Y. 2013. "Study of the rifampinmonoresistance mechanism in *Mycobacterium tuberculosis*." *Antimicrob. Agents Chemother.* 57(2) : 893-900.

Perdigão, J. Macedo, R. Malaquias, A. Ferreira, A. Brum, L. and Portugal, I. 2010. "Genetic analysis of extensively drug-resistant *Mycobacterium tuberculosis* strains in Lisbon, Portugal." *J. Antimicrob. Chemother.* 65(2) : 224-227.

Piddock, L.J. 2006. "Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria." *Clin. Microbiol. Rev.* 19(2) : 382-402.

Poole, K. 2002. "Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria." *Curr. Pharm. Biotechnol.* 3(2) : 77-98.

Prammananan, T. Cheunoy, W. Taechamahapun, D. Yorsangsukkamol, J. Phunpruch, S. Phdarat, P. Leechawengwong, M. and Chaiprasert, A. 2008. "Distribution of *rpoB* mutations among multidrug-resistant *Mycobacteriun tuberculosis* (MDRTB) strains from Thailand and development of a rapid method for mutation detection." *Clin. Microbiol. Infect.* 14(5) : 446-453.

Putman, M. Veen, H.W.V. and Konings, W.N. 2000. "Molecular properties of bacterial multidrug transporters." *Microbiol. Mol. Biol. Rev.* 64(4) : 672-693.

Plinke, C. Rüsch-Gerdes, S. and Niemann, S. 2006. "Significance of mutations in *embB* codon 306 for prediction of ethambutol resistance in clinical *Mycobacterium tuberculosis* isolates." *Antimicrob. Agents Chemother.* 50(5) : 1900-1902.

Quémard, A. Sacchettini, J.C. Dessen, A. Vilcheze, C. Bittman, R. Jacobs, W.R. Jr. and Blanchard, J.S. 1995. "Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*." *Biochemistry.* 34(26) : 8235-8241.

Ramaswamy, S. and Musser, J.M. 1998. "Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update." *Tuber. Lung Dis.* 79(1) : 3-29.

Ramirez, M.S. and Tolmasky, M.E. 2010. "Aminoglycoside modifying enzymes." *Drug Resist. Updat.* 13(6) : 151-171.

Ramón-García, S. Martin, C. Rossi, E.D. and Aínsa, J.A. 2007. "Contribution of the Rv2333c efflux pump (the Stp protein) from *Mycobacterium tuberculosis* to intrinsic antibiotic resistance in *Mycobacterium bovis* BCG." *J. Antimicrob. Chemother.* 59(3) : 544-547.

Rawat, R. Whitty, A. and Tonge, P.J. 2003. "The isoniazid-NAD adduct is a slow, tight-binding inhibitor of *InhA*, the *Mycobacterium tuberculosis* enoyl reductase: adduct affinity and drug resistance." *Proc. Natl. Acad. Sci. U.S.A.* 100(24) : 13881-13886.

Reevs, A.Z. Campbell, P.J. Sultana, R. Malik, S. Murray, M. Pikaytis, B.B. Shinnick, T.M. and Posey, J.E. 2013. "Aminoglycoside cross-resistance in *Mycobacterium tuberculosis* due to mutations in the 5′ untranslated region of *whiB7*." *Antimicrob. Agents Chmother.* 57(4) : 1857-1865.

Regmi, S.M. Chaiprasert, A. Kulawonganunchai, S. Tongsima, S. Coker, O.O. Prammananan, T. Viratyosin, W. and Thaipisuttikul, I. 2015. "Whole genome sequence analysis of multidrug-resistant *Mycobacterium tuberculosis* Beijing isolates from an outbreak in Thailand." *Mol. Genet. Genomics.* 290(5) : 1933-1941.

Ríbón, W. 2013. **Biochemical isolation and identification of mycobacteria.** Rijeka : Intech.

Rozwarski, D.A. Grant, G.a. Barton, D.H. Jacobs, W.R. Jr., and Sacchettini, J.C. 1998. "Modification of the NADH of the isoniazid target (*InhA*) from *Mycobacterium tuberculosis*." *Science.* 279(5347) : 98-102.

Safi, H. Lingaraju, S. Amin, A. Kim, S. Jones, M. Holmes, M. McNeil, M. Peterson, S.N. Chatterjee, D. Fleischmann, R. and Alland, D. 2013. "Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-ß-D-arabinosee biosynthetic and utilization pathway genes." *Nat. Genet.* 45(10) : 1190-1197.

Sakula, A. 1982. "Robert Koch: centenary of the discovery of the tubercle bacillus, 1882." *Thorax.* 37(4) : 246-251.

Sarathy, J.P. Dartois, V. and Lee, E.J.D. 2012. "The role of transport mechanisms in *Mycobacterium tuberculosis* drug resistance and tolerance." *Pharmaceuticals (Basel).* 5(11) : 1210-1235.

Scorpio, A. and Zhang, Y. 1996. "Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus." *Nat. Med.* 2(6) : 662-667.

Sensi, P. 1983. "History of the development of rifampin." *Rev. Infect. Dis.* 3 : 402-406.

Sensi, P. 1960. "Rifomycin. I. Isolation and properties of rifomycin B and rifomycin complex." *Antibiot. Annu.* 7 : 262-270.

Sherman, D.R. Mdluli, K. Hickey, M.J. Barry, C.E. 3rd. and Stover, CK. 1999. "AhpC, oxidative stress and drug resistance in *Mycobacterium tuberculosis*." *Biofactors.* 10(2-3) : 211-217.

Shi, W. Zhang, X. Jiang, X. Yuan, H. Lee, J.S. Barry, C.E. 3rd. Wang, H. Zhang, W. and Zhang, Y. 2011. "Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*." *Science.* 333(6049) : 1630-1632.

Shinnick, T. 1996. **Current Topics in Microbiology and Immunology.** New York : Springer-Verlag.

Siddiqi, N. Shamim, M. Hussain, S. Choudhary, R.K. Ahmed, N. Banerjee, S. Savithri, G.R. Alam, M. Pathak, N. Amin, A. Hanief, M. Katoch, V.M. Sharma, S.K. and Hasnain, S.E. 2004. "Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India." *Antimicrob. Agents Chemother.* 46(2) : 443-450.

Sirgel, F.A. Tait, M. Warren, R.M. Streicher, E.M. Böttger, E.C. Van Helden, P.D. Gey Van Pittius, N.C. Coetzee, G. Hoosain, E.Y. Chabula-Nxiweni, M. Hayes, C. Victor, T.C. and Trollip, A. 2012. "Mutations in the *rrs* A1401G gene and phenotypic resistance to amikacin and capreomycin in *Mycobacterium tuberculosis*." *Microb. Drug Resist.* 18(2) : 193-197.

Suzuki, Y. Katsukawa, C. Tamaru, A. Abe, C. Makino, M. Mizuguchi, Y. and Taniguchi, H. 1998. "Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene." *J. Clin. Microbiol.* 36(5) : 1220-1225.

Sirgel, F.A. Wiid, I.J.F. and Van Helden, P.D. 2009. **Measuring minimum inhibitory concentrations in mycobacteria.** New York : Human Press.

Snewin, V.A. Gares, M.P. Ó Gaora, P. Hasan, Z. Brown, I.N. and Young, D.B. 1999. "Assessment of immunity to mycobacterial infection with luciferase reporter constructs." *Infect. Immun.* 67(9) : 4586-4593.

Sreevatsan, S. Pan, X. Stockbauer, K.E. Williams, D.L. Kreiswirth, B.N. and Musser, M. 1996. "Characterization of *rpsL* and *rss* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities." *Antimicrob. Agents Chemother.* 40(4) : 1024-1026.

Takayama, K. and Kiburn, J.O. 1989. "Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*." *Antimicrob. Agents Chemother.* 33(9) : 1493-1499.

Takiff, H.E. Salazar, L. Guerrero, C. Philipp, W. Huang, W.M. Kreiswirth, B. Cole, S.T. Jacobs, W.R. Jr. and Telenti, A. 1994. "Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations." *Antimicrob. Agents Chemother.* 38(4) : 773-380.

Tamma, P.D. Cosgrove, S.E. and Maragakis, L.L. 2012. "Combination therapy for treatment of infections with gram-negative bacteria." *Clin. Microbiol. Rev.* 25(3) : 450-470.

Telenti, A. lamboden, P. Marchesi, F. Lowrie, D. Cole, S. Colston, M.J. Matter, L. Schopfer, K. and Bodmer, T. 1993. "Detection of rifampin-resistance mutations in *Mycobacterium tuberculosis*." *Lancet.* 38(4) : 773-380.

Telenti, A. Philipp, W.J. Sreevatsan, S. Bernasconi, C. Stockbauer, K.E. Wieles, B. Musser, J.M. and Jacobs, W.R. Jr. 1997. "The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol." *Nat. Med.* 3(5) : 567-570.

Thee, S. Detjen, A. Quarcoo, D. Wahn, U. and Magdorf, K. 2007. "Ethambutol in paediatric tuberculosis: aspects of ethambutol serum concentration, efficacy and toxicity in children." *Int. J. Tuberc. Dis.* 11(9) : 965-971.

Thorvaldsdóttir, H. Robinson, J.T. and Mesirov, J.P. 2013. "Integrative genomics viewer (IGV): high-performance genomics data visualization and exploration." *Brief Bioinform.* 14(2) : 178-192.

Thompson, J.D. and Higgins, D.G. and Gibson, T.J. 1994. "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic. Acids Res.* 22(22) : 4673-4680.

Thorel, M.F. Karoui, C. Varnerot, A. Fleury, C. and Vincent, V. 1998. "Isolation of *Mycobacterium bovis* from baboons, leopards and a seal-ion." *Vet. Res.* 29(2) : 207-212.

Timmins, G.S. and Deretic, V. 2006. "Mechanisms of action of isoniazid." *Mol. Microbiol.* 62(5) : 1220-1227.

Umezawa, H. 1958. "Kanamycin: its discovery." *Ann. N. Y. Acad. Sci.* 76(2) : 20-26.

Vetting, M.W.S. De Carvalho, L.P. Yu, M. Hegde, S.S. Magnett, S. Roderick, S.L. and Blanchard, J.S. 2005. "Structure and functions of the GNAT superfamily of acetyltransferases." *Arch. Biochem. Biophys.* 433(1) : 212-226.

Villellas, C. Aristimuno, L. Vitoria, M.A. Prat, C. Blanco, S. Viedma, D.G. Domínguez, J. Samper, S. and Ainsa, J.A. 2013. "Analysis of mutations in streptomycin-resistant strains reveals a simple and reliable genetic marker for identification of the *Mycobacterium tuberculosis* Beijing genotype." *J. Clin. Microbiol.* 51(7) : 2124-2130.

Von Groll, A. Marti, A. Jureen, P. Hoffner, S. Vandamme, P. Protaels, F. Palomino, J.C. and Da Silva, P.A. 2009. "Fluoroquinolone resistance in *Mycobacterium tuberculosis* and mutations in *gryA* and *gryB*." *Antimicrob. Agents Chemother.* 53(10) : 4498-4500.

WHO. 2015. "Global tuberculosis report 2015." 1-48. in A short update to the 2015 report. **WHO.** Geneva : World Health Organization.

Wolfson, J.S. and Hooper, D.C. 1985. "The fluoroquinolones: structure, mechanisms of action and resistance, and spectra of activity in vitro." *Antimicrob. Agents Chemother.* 28(4) : 581-586.

Wong, S.Y. Lee, J.S. Kwak, H.K. Via, L.E. Boshoff, H.I. and Barry, C.E. 3rd. 2011. "Mutations in *gidB* confer low-level streptomycin resistance in *Mycobacterium tuberculosis*." *Am. Rev. Tuberc.* 65(5) : 523-46.

Woodruff, C.E. Crobie, D. Woolley, J.S. Woolley, E. and Steenken, W. 1946. "Committee on evaluation of laboratory procedure." *Am. Rev. Tuberc.* 54 : 428-132.

Yeager, R.L. Munroe, W.G. and Dessay, F.I. 1952. "Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis." *Am. Rev. Tuberc.* 65(5) : 523-46.

Yin, H. Litvinov, R.I. Vilaire, G. Zhu, H. Li, W. Caputo, G.A. Moore, D.T. Lear, J.D. Weisel, J.W. Degrado, W.F. and Bennett, J.S. 2006. "Activation of platelet alphallbbeta 3 an exogenous peptide corresponding to the transmembrane domain of alphalb." *J. Biol. Chem.* 281(48) : 36732--36741.

Yuan, X. Zhang, T. Kawakami, K. Zhu, J. Li, H. Lei, J. and Tu, S. 2012. "Molecular characterization of multidrug- and extensively drug-resistant *Mycobacterium tuberculosis* strains in Jiangxi China." *J. Clin. Microbiol.* 50(7) : 2404-2413.

Zaunbrecher, M.A. Sikes, R.D. Metchock, B. Shinnick, T.M. and Posey, J.E. 2009. "Overexpression of the chromosomally encoded aminoglycosdie acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*." *Proc. Nat. Acad. Sci. USA.* 196(47) : 20004-20009.

Zhang, Y. Heym, B. Allen, B. Young, D. and Cole, S. 1992. "The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*." *Nature.* 358(6387) : 591-593.

Zhang, Y. and Mitchison, D. 2003. "The curious characteristics of pyrazinamide: a review." *Int. J. Tuberc. Lung Dis.* 7(1) : 6-21.

Zhang, Y. and Yew, W.W. 2009. "Mechanisms of drug resistance in *Mycobacterium tuberculosis*." *Int. J. Tuberc. Lung Dis.* 13(11) : 1320-1330.

[Online]. Available: http://www.eac.int/health/images/tb/transmission.jpg

[Online]. Available: http://www.medwow.com/med/microbiological-culture-analyzer/becton-dickinson/bactec-460-tb-system/xbactec-460-tb-system.mth14330_200_200.jpg.pagespeed.ic.CiTe828r31.jpg

[Online]. Available: http://static.framar.bg/thumbs/6/atc/Isoniazid_11.jpg

[Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2866272/figure/fig02/

[Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2866272/figure/fig01/

[Online]. Available: https://www.drugbank.ca/structures/DB01045/image.svg

[Online]. Available: https://www.drugbank.ca/structures/DB00339/image.png

[Online]. Available: https://www.drugs.com/img/mol/DB00330.mol.png

[Online]. Available: http://cdn.biologydiscussion.com/wp-content/uploads/2016/09/clip_image002-134.jpg

[Online]. Available: http://aac.asm.org/content/48/3/799/F1.large.jpg

[Online]. Available: http://textbookofbacteriology.net/MTBCDC.jpg

[Online]. Available: https://www.askjpc.org/wsco/wsc/images/2012/121102-3.jpg

[Online]. Available: http://www.nature.com/nrmicro/journal/v12/n8/images/nrmicro3299-i1.jpg

[Online]. Available: http://www.uaz.edu.mx/histo/pathology/ed/ch_9b/c9b_mtb_mac.jpg

[Online]. Available: http://4.bp.blogspot.com/-iPcDg7yVP8o/UGpXK9AlelI/AAAAAAAAFkk/h_BEg72JDlE/s524/08-Neomyicin-B-%2528Framycetin%2529.jpg

[Online]. Available: http://www.cell.com/cms/attachment/2016508716/2037209025/fx1.jpg

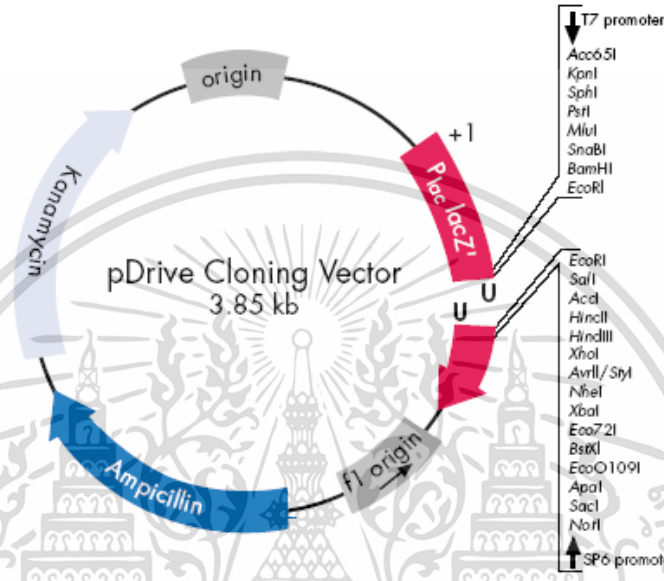
[Online]. Available: https://www.researchgate.net/profile/Joel_Pedersen/publication/26267802/figure/fig3/AS:310096199471106@1450944103273/Fig-3-Chemical-structure-of-kanamycin-tobramycin-and-amikacin-Grey-region-shows-the.png

[Online]. Available: http://image.shutterstock.com/z/stock-photo-mechanism-of-antibiotic-resistance-in-bacteria-labeled-diagram-166170113.jpg

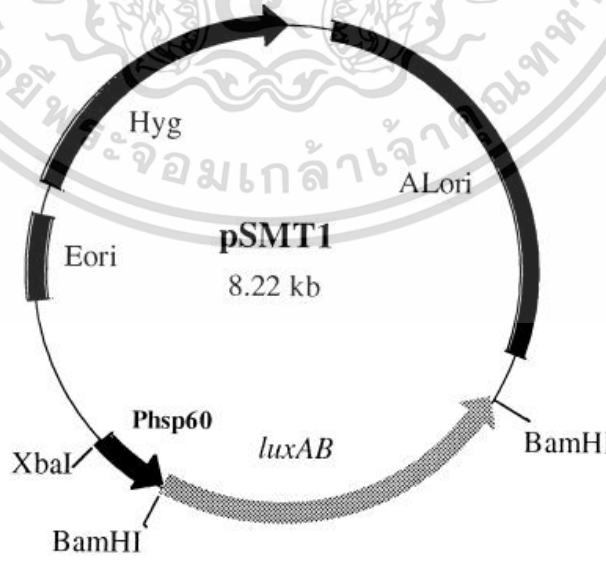
Appendices

APPENDIX A

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



2. Mapping gene of pSMT1 vector (Snewin *et al.*, 1999)



APPENDIX B

1. Luria-Bertani (LB) medium

LB broth

Ingredients (g/L) :	Tryptone	10
	Yeast extract	5
	Sodium chloride	10

The 15 g/L of agar was additionally added for LB agar. The pH of media was adjusted to 7.4 by NaOH and autoclved at 121 °C for 15 min.

2. SOB medium

Ingredients (g/L) :	Tryptone	20.0
	Yeast extract	5.0
	Sodium chloride	0.5

The pH of media was adjusted to 7.5 by NaOH and autoclved at 121 °C for 15 min.

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

OADC was sterilized in autoclve at 121 °C for 15 min.

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

OADC was sterilized in autoclve at 121 °C for 15 min.

4. Middlebrook 7H9 and 7H10 medium

One liter of medium were prepare by suspend 19 g of Middlebrook 7H9 or 7H10 powder in 850 ml of sterile water. Five milliliters of glycerol were added and made up the volume with water up to 900 ml. Agar was added in Middlebrook 7H10. The media was sterilized in autoclave at 121 °C for 15 min, and add 100 ml of OADC.

Ingredients (g/L) :	Ammonium sulfate	0.50
	L-Glutamic acid	0.50
	Monopotassium phosphate	1.50
	Disodium phosphate	1.50
	Ferric ammonium citrate	0.40
	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 (g/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 sterilization by filter 25 mm.

6. RF2 solution

Ingredients (g/L) :

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

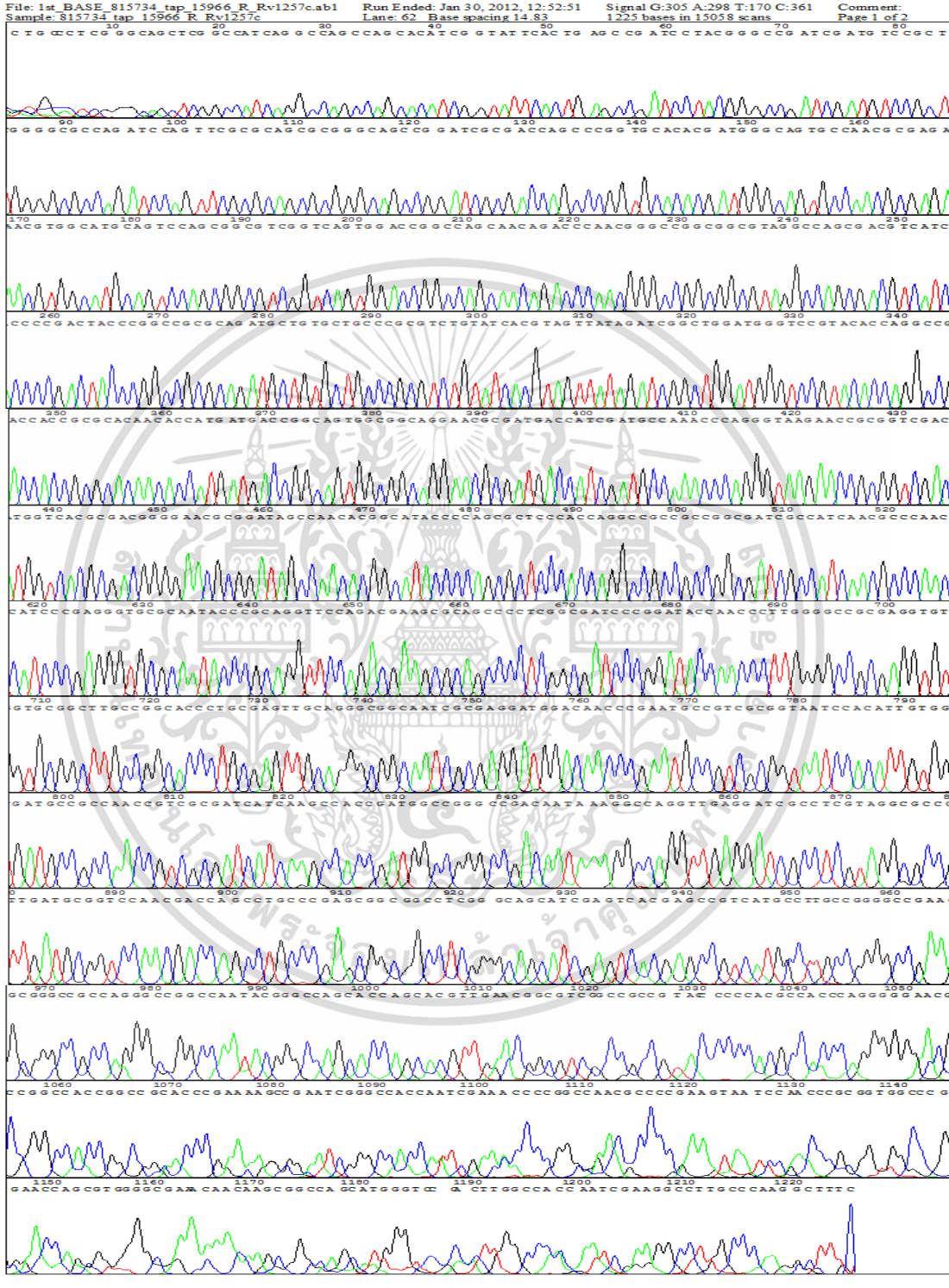
The pH was adjusted to 6.8 by NaOH and sterilization by filter 25 mm.

APPENDIX C

1. Chromatogram of 16S rRNA (*rrs*) nucleotide sequence

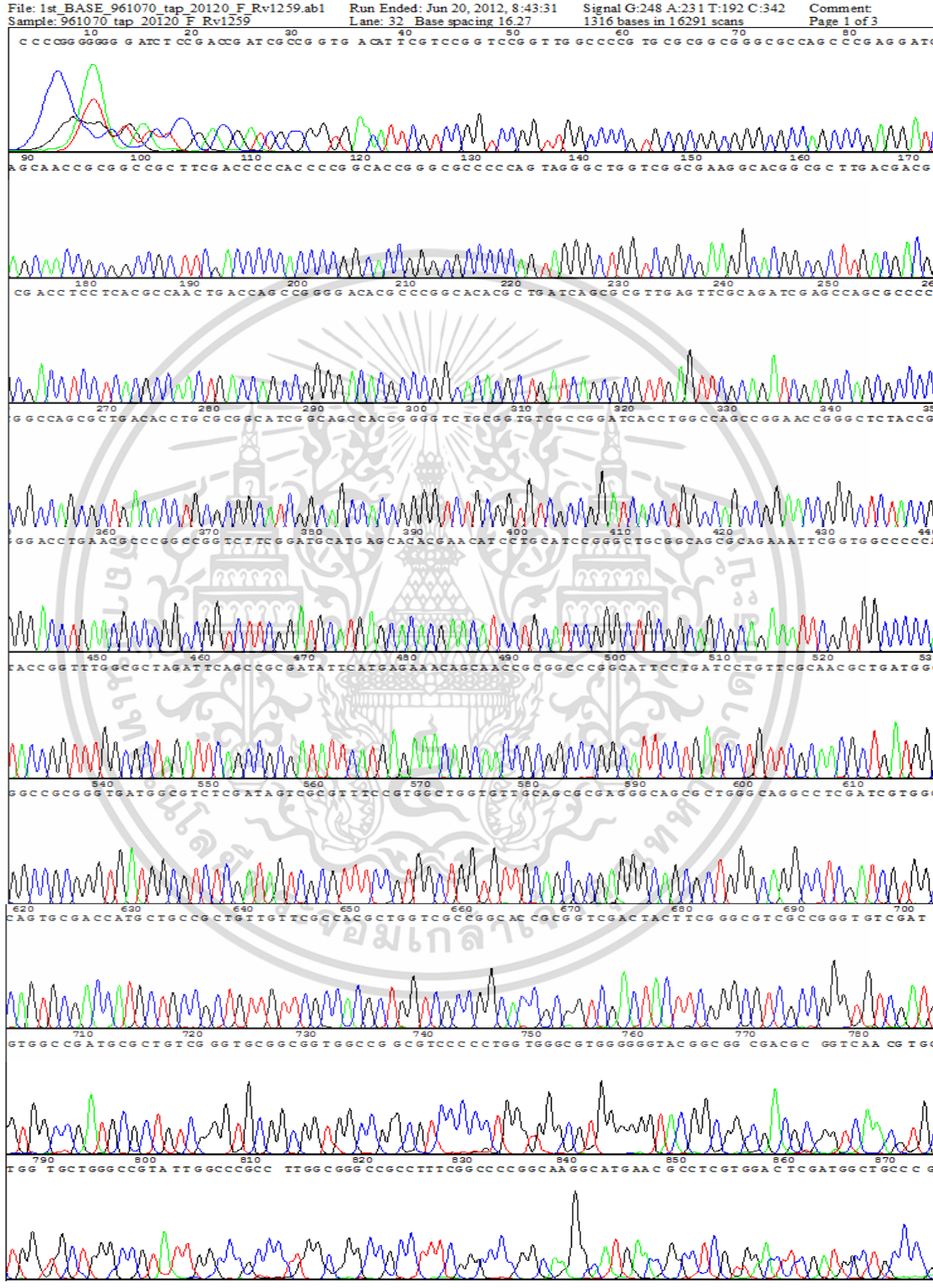
APPENDIX C (CONTINUED)

2. Chromatogram of *eis* promoter nucleotide sequence



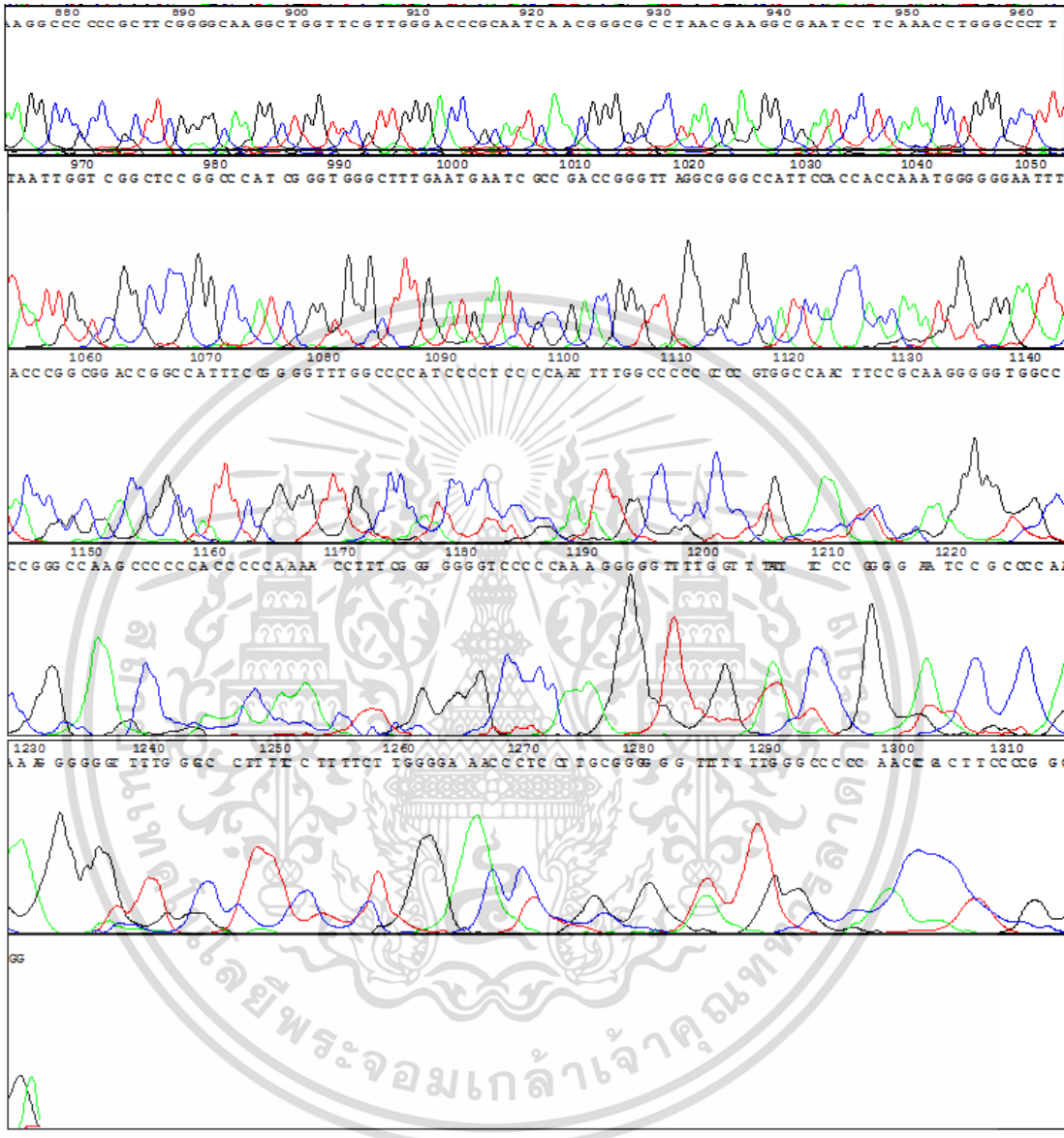
APPENDIX C (CONTINUED)

3. Chromatogram of *tap* nucleotide sequence



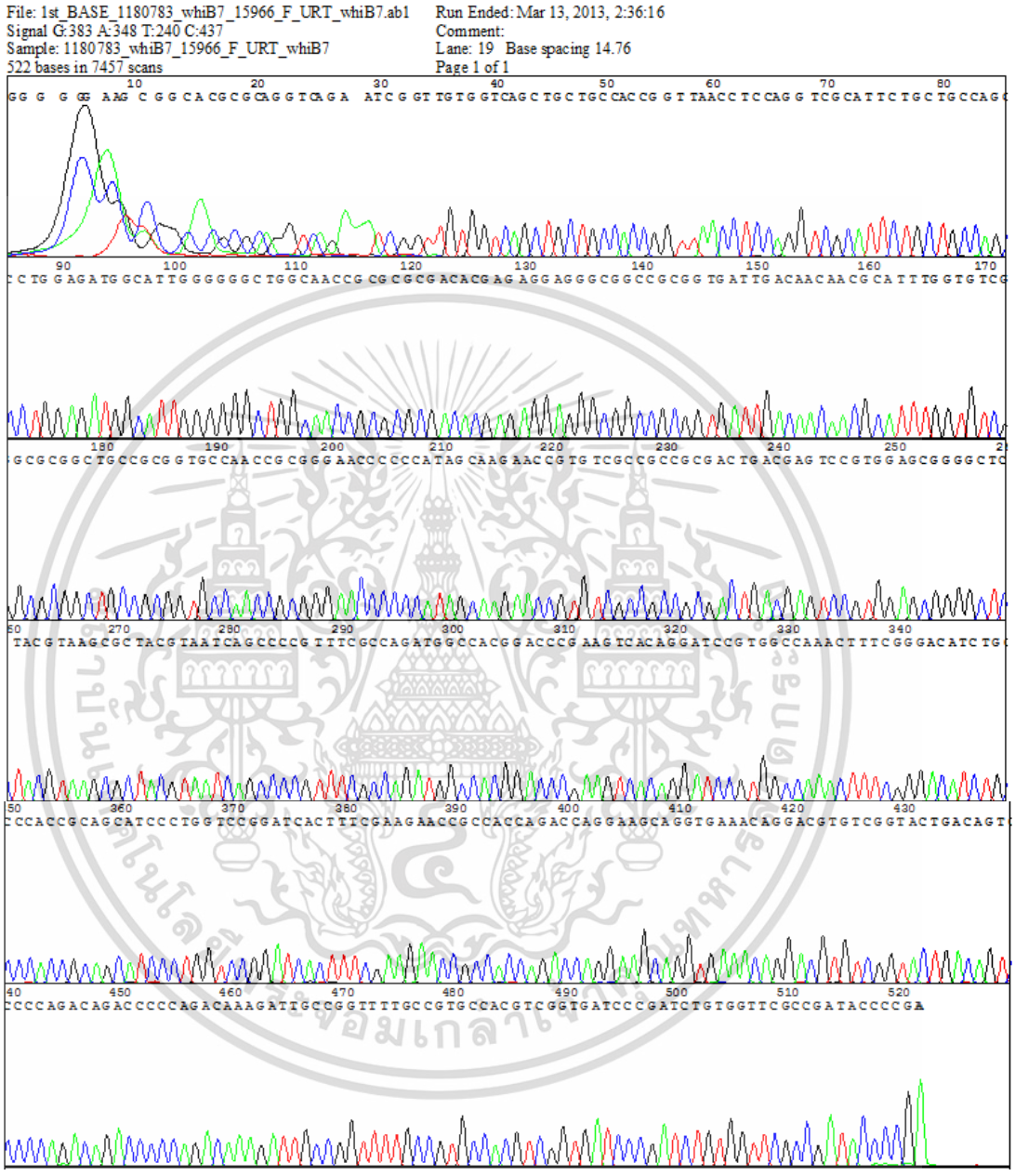
APPENDIX C (CONTINUED)

3. Chromatogram of *tap* nucleotide sequence (continued)



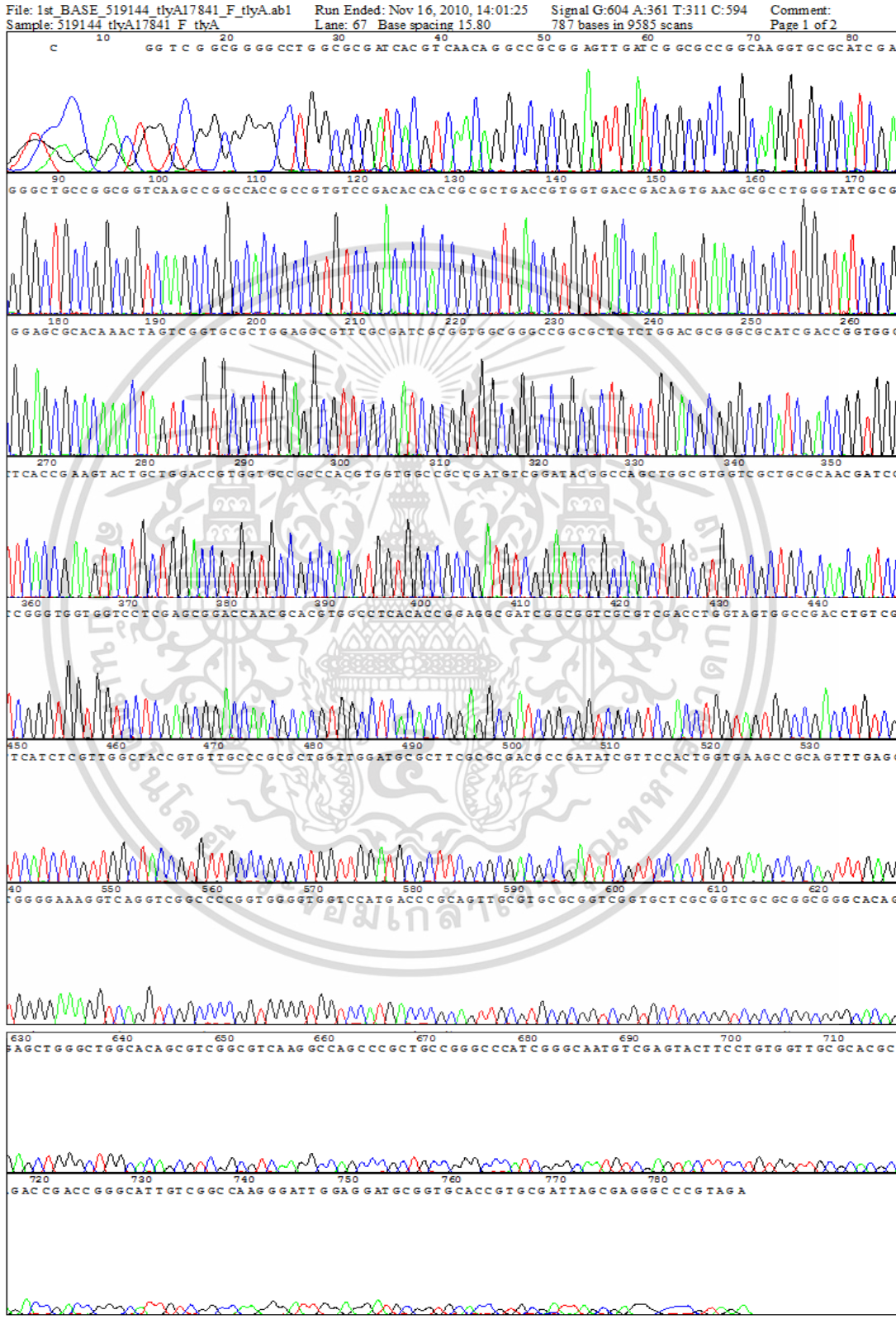
APPENDIX C (CONTINUED)

4. Chromatogram of *whiB7* nucleotide sequence



APPENDIX C (CONTINUED)

5. Chromatogram of *tlyA* nucleotide sequence



APPENDIX D

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
1977	.	A	G	206339	Rv0174_1109	T	C
4013	Rv0003_734	T	C	223942	Rv0192_379	T	C
7362	Rv0006_61	G	C	225323	Rv0193c_1249	T	C
7585	Rv0006_284	G	C	227098	Rv0194_221	T	C
9304	Rv0006_2003	G	A	231114	Rv0195_216	C	G
11879	Rv0008c_433	A	G	234477	Rv0197_2247	T	G
14785	Rv0012_697	T	C	261869	Rv0218_946	T	C
21795	Rv0018c_1387	G	A	265554	Rv0222_48	A	C
26959	.	C	G	278681	Rv0233_97	C	G
34044	.	T	C	285772	Rv0236c_1080	A	C
37031	Rv0034_165	C	G	285871	Rv0236c_981	A	G
42967	Rv0040c_399	G	C	310973	Rv0259c_545	G	A
62049	Rv0058_1654	A	G	311613	Rv0260c_1047	G	T
69989	Rv0064_1370	G	A	346275	Rv0284_641	C	G
70816	Rv0064_2197	A	G	356528	Rv0292_649	A	G
71336	Rv0064_2717	G	C	376774	Rv0307c_282	T	C
75940	Rv0068_640	G	C	384380	Rv0315_779	A	C
92199	Rv0083_1800	T	G	386432	Rv0318c_668	C	G
116000	Rv0101_6000	T	G	390828	Rv0323c_424	T	C
122109	Rv0103c_65	A	G	403980	Rv0338c_1862	G	A
125830	.	GA	GAA	404326	Rv0338c_1516	T	C
133839	.	C	T	414486	Rv0344c_456	C	T
146087	Rv0120c_1685	T	C	420008	Rv0350_174	A	G
154283	Rv0127_52	T	C	454295	Rv0376c_78	T	C
194681	Rv0165c_258	G	C	457452	Rv0381c_372	T	G
196642	Rv0166_1650	C	T	459399	.	A	C

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
475178	Rv0395_239	T	C	841764	Rv0749A_111	G	C
489935	Rv0405_4205	G	C	852910	Rv0758_515	C	T
498557	Rv0412c_1063	C	A	857696	Rv0764c_342	A	G
502589	Rv0417_224	C	G	874835	.	CC	CCGC
503354	.	G	C	880562	Rv0785_1223	G	T
513257	Rv0425c_2065	T	C	882257	Rv0787_799	T	C
541201	Rv0450c_291	A	G	893733	Rv0800_416	T	G
551525	Rv0459_328	A	C	900221	Rv0806c_1110	T	C
573262	Rv0484c_540	A	G	903550	Rv0808_1440	T	C
590436	Rv0500_354	T	C	903913	Rv0809_189	T	C
597816	Rv0507_618	A	G	906857	Rv0812_435	A	G
598475	Rv0507_1277	G	A	945214	Rv0848_277	G	A
610120	.	T	G	949535	Rv0853c_1584	T	C
630722	Rv0538_683	G	C	955524	Rv0859_448	A	G
637319	Rv0545c_145	G	A	979704	Rv0881_343	G	C
665293	Rv0572c_91	A	G	986463	.	G	C
669398	Rv0575c_348	T	C	993346	Rv0891c_110	A	C
685461	Rv0587_333	C	G	1010204	.	CG	CGG
685608	Rv0587_480	T	C	1025106	Rv0919_423	T	C
686972	Rv0589_152	T	C	1037012	Rv0930_14	T	C
690465	Rv0591_1407	T	G	1037911	Rv0930_913	C	T
698968	Rv0601c_27	G	A	1047165	Rv0938_1030	T	C
754186	Rv0658c_224	A	G	1068151	Rv0956_591	T	C
775639	Rv0676c_2842	T	C	1068432	Rv0957_228	A	G
781395	.	T	C	1070702	Rv0958_820	T	C
820483	Rv0727c_17	G	T	1074558	Rv0962c_557	G	A

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
1075279	.	T	C	1327890	Rv1186c_1416	G	A
1076309	Rv0964c_370	G	T	1328687	Rv1186c_619	G	C
1077312	Rv0966c_524	A	G	1374065	Rv1230c_133	T	C
1079927	Rv0969_1185	C	A	1375724	Rv1232c_445	A	C
1081681	Rv0970_630	T	C	1382628	Rv1239c_415	T	C
1087193	Rv0974c_153	G	C	1393626	Rv1249c_357	A	G
1096633	.	T	G	1396922	Rv1251c_2319	T	C
1100234	Rv0983_1169	T	C	1411210	Rv1263_780	T	G
1106422	Rv0989c_961	T	C	1413148	.	C	T
1109975	Rv0993_704	A	G	1414021	Rv1266c_1820	C	T
1126889	Rv1007c_115	G	C	1440469	Rv1286_1563	C	G
1127648	Rv1008_560	C	A	1445781	Rv1291c_54	A	G
1149551	Rv1028c_2136	C	T	1457144	Rv1300_580	C	T
1150585	Rv1028c_1102	G	A	1471659	.	C	T
1168715	.	CT	CTT	1482627	Rv1320c_1591	T	C
1178116	Rv1056_489	T	C	1484708	Rv1321_430	A	C
1200418	.	A	G	1499274	Rv1330c_1286	C	G
1220680	Rv1093_107	T	C	1526819	Rv1358_208	C	A
1224367	.	T	C	1536251	Rv1364c_1394	G	T
1248978	Rv1125_897	T	C	1547125	Rv1374c_406	T	C
1276588	.	C	G	1552547	Rv1378c_109	G	A
1281118	Rv1154c_367	T	C	1563717	Rv1388_24	C	T
1292102	Rv1162_1038	A	G	1570566	Rv1394c_404	C	A
1307598	Rv1175c_629	C	G	1588899	Rv1412_333	G	T
1315191	Rv1180_1467	A	C	1609840	Rv1431_1758	A	G
1315884	Rv1181_651	G	A	1613035	.	T	C

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
1624791	Rv1446c_575	C	G	1917972	Rv1694_33	A	G
1630148	Rv1449c_52	A	C	1931179	Rv1704c_278	C	A
1639594	Rv1453_1214	C	A	1933988	.	G	A
1645802	Rv1459c_337	T	C	1944107	Rv1716_532	A	G
1650072	Rv1462_547	A	G	1944402	Rv1716_827	T	C
1676290	Rv1486c_594	C	A	1950767	Rv1724c_285	T	C
1689349	Rv1498c_572	C	T	1960284	Rv1733c_204	C	A
1692141	Rv1501_252	A	C	1967237	Rv1739c_401	C	A
1693561	Rv1502_638	A	G	2022868	Rv1784_2235	T	C
1698911	Rv1508c_984	G	A	2055271	Rv1812c_89	A	G
1706119	Rv1514c_477	T	C	2057774	Rv1815_247	A	T
1728837	.	A	G	2096186	Rv1846c_414	A	G
1759252	Rv1552_1572	G	T	2108141	Rv1860_406	T	C
1760292	Rv1554_118	A	G	2116903	Rv1867_1140	C	T
1778430	Rv1570_572	T	C	2123169	.	T	G
1798355	Rv1597_62	G	A	2128870	Rv1878_849	A	G
1803265	Rv1602_602	G	A	2135870	.	T	C
1804409	Rv1604_371	C	A	2143328	Rv1895_808	G	C
1817976	Rv1618_362	A	T	2147022	Rv1900c_612	A	C
1836286	.	G	C	2207591	.	TT	TCT
1847919	Rv1639c_540	C	G	2211826	Rv1968_201	A	G
1854300	Rv1644_695	T	C	2216443	Rv1971_1187	C	A
1856777	Rv1647_4	G	C	2220512	Rv1977_759	T	G
1885772	Rv1662_4069	G	A	2223293	.	T	C
1901493	Rv1676_447	T	C	2228967	.	A	G
1907296	Rv1682_894	G	C	2251999	.	A	G

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
2260525	.	C	T	2509140	Rv2236c_236	G	C
2264782	Rv2017_785	C	A	2509722	Rv2237_234	A	G
2265059	.	T	G	2521342	Rv2247_600	T	C
2269780	Rv2024c_461	T	C	2525722	.	CG	C
2270102	Rv2024c_139	A	G	2529680	Rv2256c_195	A	G
2282787	Rv2037c_935	C	T	2531742	Rv2258c_156	A	G
2285251	Rv2039c_391	C	A	2534562	.	GGA	G
2287121	Rv2041c_726	A	G	2573756	.	C	A
2300237	Rv2048c_6750	A	G	2586127	Rv2314c_1164	A	G
2300546	Rv2048c_6441	A	T	2598400	Rv2326c_1548	A	G
2329533	Rv2072c_614	A	G	2612632	Rv2337c_356	C	A
2334007	Rv2077c_288	A	G	2626513	.	T	A
2335075	Rv2078_17	A	G	2656225	Rv2377c_206	A	G
2335494	Rv2079_140	A	G	2660319	Rv2379c_1767	C	G
2340621	Rv2082_1913	C	G	2680658	.	T	G
2341636	Rv2083_766	C	G	2695378	Rv2398c_422	C	G
2345037	Rv2088_627	C	A	2713795	.	C	T
2348446	Rv2090_1074	C	G	2718852	.	T	G
2361604	Rv2101_1365	C	G	2734074	Rv2436_845	T	C
2362041	Rv2101_1802	C	A	2751804	Rv2450c_377	C	T
2368564	.	TA	T	2752698	.	C	A
2386389	Rv2125_97	G	A	2760152	Rv2458_374	A	G
2415656	Rv2155c_739	G	C	2779136	Rv2476c_3127	T	C
2424925	.	A	G	2786952	Rv2482c_2332	A	G
2462871	Rv2198c_177	G	A	2809621	Rv2495c_319	T	C
2499726	Rv2226_895	G	A	2818837	Rv2503c_291	A	G

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
2821342	Rv2505c_255	C	T	3080795	Rv2771c_239	A	G
2827984	.	G	T	3086788	.	T	C
2830525	Rv2513_365	C	A	3103682	Rv2794c_259	T	C
2855259	Rv2531c_2523	A	G	3113872	Rv2807_215	A	T
2865760	Rv2542_631	A	G	3133536	Rv2825c_4	T	C
2865882	Rv2542_753	T	C	3137058	Rv2830c_167	G	A
2880702	Rv2560_628	G	C	3177884	Rv2866_63	C	A
2881597	.	AG	A	3186860	Rv2874_2014	T	G
2888201	Rv2566_1829	T	C	3189523	.	C	T
2889633	Rv2566_3261	T	C	3190145	.	TC	T
2891267	Rv2567_1473	C	T	3226181	Rv2916c_105	A	C
2891728	Rv2567_1934	A	G	3228143	Rv2917_1781	G	T
2894208	Rv2569c_201	G	A	3247316	Rv2931_1872	C	G
2910461	Rv2584c_440	G	T	3247851	Rv2931_2407	G	A
2911293	Rv2585c_1385	C	G	3248074	Rv2931_2630	G	A
2912294	Rv2585c_384	T	G	3256494	Rv2933_810	A	G
2923391	Rv2592c_843	T	C	3269581	Rv2935_1845	A	G
2927939	.	T	C	3270784	Rv2935_3048	A	G
2939373	Rv2611c_590	G	C	3296843	Rv2947c_998	A	G
2939657	Rv2611c_306	T	C	3308606	.	G	A
2954439	Rv2627c_310	T	C	3336825	Rv2981c_1093	T	C
2984740	Rv2668_8	A	G	3338603	Rv2982c_397	G	C
3005185	Rv2688c_466	G	T	3358235	Rv2999_634	A	T
3009692	Rv2691_349	A	G	3363338	.	A	G
3017465	Rv2702_608	T	C	3367765	Rv3009c_1029	G	A
3041871	Rv2729c_605	G	T	3402816	Rv3042c_347	C	T

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT	Position	Genes	Ref	ALT
3425854	Rv3062_271	C	T	3817117	Rv3399_989	C	A
3428917	Rv3063_1675	C	A	3823159	Rv3403c_705	A	T
3440464	Rv3077_924	T	G	3826684	Rv3408_137	C	T
3456666	Rv3089_906	A	G	3829770	Rv3410c_141	T	C
3462135	Rv3093c_630	G	C	3838871	Rv3420c_192	A	G
3473996	.	GA	GAA	3859893	Rv3440c_84	C	T
3486977	Rv3121_469	A	G	3862472	.	GA	G
3503895	Rv3137_503	C	T	3864995	Rv3447c_3244	T	C
3518167	Rv3151_1422	A	G	3877421	Rv3456c_12	A	G
3518555	Rv3151_1810	A	G	3884906	Rv3467_943	A	G
3556275	Rv3190c_413	A	G	3885886	Rv3468c_184	T	C
3580636	.	CT	C	3892671	Rv3476c_1050	A	G
3581414	Rv3204_100	A	G	3896340	Rv3479_521	T	G
3590686	.	GT	GCT	3898408	Rv3479_2589	A	G
3591063	Rv3213c_430	T	C	3952800	Rv3516_257	G	A
3604821	Rv3228_96	G	C	3958403	Rv3521_883	A	G
3614982	Rv3239c_2622	T	C	3959418	Rv3522_971	C	T
3622441	Rv3243c_651	A	C	4005607	Rv3564_361	T	C
3625065	Rv3245c_1549	T	G	4024273	Rv3581c_75	T	C
3689523	Rv3303c_1416	G	T	4026899	Rv3585_456	G	A
3704596	Rv3317_160	G	C	4034827	Rv3593_476	C	T
3714211	Rv3328c_122	G	T	4055801	Rv3616c_575	G	A
3714757	Rv3329_366	A	C	4059904	.	A	G
3718357	Rv3331_1268	C	T	4069292	Rv3630_118	G	A
3721806	Rv3335c_795	G	C	4100975	.	T	C
3798095	Rv3383c_395	A	C	4111303	Rv3669_477	G	C

APPENDIX D (CONTINUED)

Table D The nucleotide alteration position of *M. tuberculosis* MT433

Position	Genes	Ref	ALT
4156099	Rv3711c_631	C	A
4162339	Rv3719_34	A	G
4182695	Rv3731_938	G	A
4187485	Rv3736_852	T	C
4187817	Rv3737_119	A	G
4197138	.	CT	CTT
4198611	.	CG	C
4204441	Rv3759c_933	A	G
4210274	Rv3764c_736	A	G
4221490	Rv3776_402	C	G
4222073	Rv3776_985	A	G
4222882	Rv3777_189	A	G
4242643	Rv3793_2781	C	T
4255922	Rv3799c_27	A	G
4257220	Rv3800c_3927	A	G
4306155	Rv3831_399	C	T
4307179	Rv3833_313	G	A
4338595	.	GC	G
4338732	.	G	A
4356110	Rv3877_1104	G	C
4366272	Rv3884c_567	G	C
4379680	Rv3894c_773	C	G
4382054	Rv3896c_798	T	C
4382275	Rv3896c_577	G	T
4400660	.	AC	A

APPENDIX E

1. Nucleotide sequence of Rv1819c of *M. tuberculosis* MT433

```
Rv1819c             ----------------------------------GGATCCTTGGGCCCGAAATTGTTTAAG
Rv1819c_MT433       CGAAGGATTTCTGTTTGTGAGGAGACGTCGACCGGATCCTTGGGCCCGAAATTGTTTAAG
                                                     **************************

Rv1819c             CCGTCCATCGATTGGTCTAGAGCATTCCCGGATTCCGTGTATTGGGTCGGCAAAGCCTGG
Rv1819c_MT433       CCGTCCATCGATTGGTCTAGAGCATTCCCGGATTCCGTGTATTGGGTCGGCAAAGCCTGG
                    ************************************************************

Rv1819c             ACGATCAGTGCCATCTGTGTGCTCGCGATATTGGTTCTGCTCAGGTATTTGACGCCCTGG
Rv1819c_MT433       ACGATCAGTGCCATCTGTGTGCTCGCGATATTGGTTCTGCTCAGGTATTTGACGCCCTGG
                    ************************************************************

Rv1819c             GGCCGGCAGTTCTGGCGGATCACCCGCGCATACTTCGTTGGCCCCAACAGCGTTCGCGTG
Rv1819c_MT433       GGCCGGCAGTTCTGGCGGATCACCCGCGCATACTTCGTTGGCCCCAACAGCGTTCGCGTG
                    ************************************************************

Rv1819c             TGGCTGATGCTCGGCGTGTTGTTGCTTTCGGTAGTGCTGGCAGTGCGCCTGAATGTGCTA
Rv1819c_MT433       TGGCTGATGCTCGGCGTGTTGTTGCTTTCGGTAGTGCTGGCAGTGCGCCTGAATGTGCTA
                    ************************************************************

Rv1819c             TTCAGCTACCAAGGCAACGACATGTACACGGCCTTGCAGAAGGCCTTCGAGGGCATCGCC
Rv1819c_MT433       TTCAGCTACCAAGGCAACGACATGTACACGGCCTTGCAGAAGGCCTTCGAGGGCATCGCC
                    ************************************************************

Rv1819c             TCTGGCGATGGCACGGTCAAACGCTCAGGCGTGCGAGGATTTTGGATGTCGATCGGGGTT
Rv1819c_MT433       TCTGGCGATGGCACGGTCAAACGCTCAGGCGTGCGAGGATTTTGGATGTCGATCGGGGTT
                    ************************************************************

Rv1819c             TTCAGCGTGATGGCCGTGCTGCACGTGACCCGGGTCATGGCCGACATCTACTTGACGCAG
Rv1819c_MT433       TTCAGCGTGATGGCCGTGCTGCACGTGACCCGGGTCATGGCCGACATCTACTTGACGCAG
                    ************************************************************

Rv1819c             CGCTTCATCATCGCCTGGAGGGTCTGGCTAACCCACCACCTCACACAAGACTGGCTCGAC
Rv1819c_MT433       CGCTTCATCATCGCCTGGAGGGTCTGGCTAACCCACCACCTCACACAAGACTGGCTCGAC
                    ************************************************************

Rv1819c             GGCAGGGCCTACTACCGAGACCTGTTCATCGACGAAACGATCGACAACCCCGACCAGCGC
Rv1819c_MT433       GGCAGGGCCTACTACCGAGACCTGTTCATCGACGAAACGATCGACAACCCCGACCAGCGC
                    ************************************************************

Rv1819c             ATTCAGCAAGACGTCGATATCTTCACCGCCGGGGGCAGGCGGCACTCCGAATGCTCCCTCC
Rv1819c_MT433       ATTCAGCAAGACGTCGATATCTTCACCGCCGGGGGCAGGCGGCACTCCGAATGCTCCCTCC
                    ************************************************************

Rv1819c             AACGGGACGGCCAGCACGCTGCTTTTCGGGGCCGTGCAGTCGATCATTTCGGTGATTTCT
Rv1819c_MT433       AACGGGACGGCCAGCACGCTGCTTTTCGGGGCCGTGCAGTCGATCATTTCGGTGATTTCT
                    ************************************************************
```

APPENDIX E (CONTINUED)

1. Nucleotide sequence of Rv1819c of *M. tuberculosis* MT433

```
Rv1819c            TTCACAGCGATCCTGTGGAATCTCTCGGGCACCCTGAATATCTTCGGCGTGTCCATCCCG
Rv1819c_MT433      TTCACAGCGATCCTGTGGAATCTCTCGGGCACCCTGAATATCTTCGGCGTGTCCATCCCG
                   ************************************************************

Rv1819c            CGCGCAATGTTCTGGACCGTGCTGGTCTATGTGTTCGTGGCCACGGTCATCTCGTTCATC
Rv1819c_MT433      CGCGCAATGTTCTGGACCGTGCTGGTCTATGTGTTCGTGGCCACGGTCATCTCGTTCATC
                   ************************************************************

Rv1819c            ATCGGGCGGCCCCTGATCTGGCTCAGCTTCCGCAATGAAAAGCTCAATGCCGCTTTCCGT
Rv1819c_MT433      ATCGGGCGGCCCCTGATCTGGCTCAGCTTCCGCAATGAAAAGCTCAATGCCGCTTTCCGT
                   ************************************************************

Rv1819c            TACGCGCTGGTTCGGCTACGCGACGCCGCCGAGGCGGTGGGTTTCTACCGCGGCGAGCGG
Rv1819c_MT433      TACGCGCTGGTTCGGCTACGCGACGCCGCCGAGGCGGTGGGTTTCTACCGCGGCGAGCGG
                   ************************************************************

Rv1819c            GTGGAAGGTACCCAGCTACAGCGGCGGTTCACGCCGGTGATCGACAATTACCGTCGCTAC
Rv1819c_MT433      GTGGAAGGTACCCAGCTACAGCGGCGGTTCACGCCGGTGATCGACAATTACCGTCGCTAC
                   ************************************************************

Rv1819c            GTTCGGCGCAGCATCGCATTCAATGGATGGAATCTGTCGGTGAGCCAGACAATTGTTCCG
Rv1819c_MT433      GTTCGGCGCAGCATCGCATTCAATGGATGGAATCTGTCGGTGAGCCAGACAATTGTTCCG
                   ************************************************************

Rv1819c            TTGCCGTGGGTCATCCAGGCGCCTCGATTATTCGCCGGGCAGATCGACTTCGGCGATGTC
Rv1819c_MT433      TTGCCGTGGGTCATCCAGGCGCCTCGATTATTCGCCGGGCAGATCGACTTCGGCGATGTC
                   ************************************************************

Rv1819c            GGGCAGACGGCGACTTCCTTCGGCAACATTCACGACTCGTTGTCGTTCTTCCGCAACAAC
Rv1819c_MT433      GGGCAGACGGCGACTTCCTTCGGCAACATTCACGACTCGTTGTCGTTCTTCCGCAACAAC
                   ************************************************************

Rv1819c            TACGACGCGTTTGCGTCCTTCCGCGCAGCAATCATCCGATTGCATGGGCTGGTCGACGCC
Rv1819c_MT433      TACGACGCGTTTGCGTCCTTCCGCGCAGCAATCATCCGATTGCATGGGCTGGTCGACGCC
                   ************************************************************

Rv1819c            AACGAGAAAGGCCGCGCCCTGCCCGCGGTCCTGACCCGACCGAGCGACGACGAGTCCGTC
Rv1819c_MT433      AACGAGAAAGGCCGCGCCCTGCCCGCGGTCCTGACCCGACCGAGCGACGACGAGTCCGTC
                   ************************************************************

Rv1819c            GAGCTCAACGACATCGAGGTGCGTACGCCTGCCGGCGATCGGTTGATCGACCCGCTCGAT
Rv1819c_MT433      GAGCTCAACGACATCGAGGTGCGTACGCCTGCCGGCGATCGGTTGATCGACCCGCTCGAT
                   ************************************************************

Rv1819c            GTGCGGCTGGACCGCGGAGGCTCGCTGGTGATCACCGGGCGTTCTGGGGCCGGCAAGACC
Rv1819c_MT433      GTGCGGCTGGACCGCGGAGGCTCGCTGGTGATCACCGGGCGTTCTGGGGCCGGCAAGACC
                   ************************************************************

Rv1819c            ACGCTGCTGCGCAGTCTGGCGGAACTGTGGCCCTACGCATCGGGGACCCTGCACCGGCCG
Rv1819c_MT433      ACGCTGCTGCGCAGTCTGGCGGAACTGTGGCCCTACGCATCGGGGACCCTGCACCGGCCG
                   ************************************************************
```

APPENDIX E (CONTINUED)

1. Nucleotide sequence of Rv1819c of *M. tuberculosis* MT433

```
Rv1819c             CGTGACGTGGTGTGCTACCCCAACTCTGCGGCCGCCATCCCCGACGCCACCCTGCGGGAC
Rv1819c_MT433       CGTGACGTGGTGTGCTACCCCAACTCTGCGGCCGCCATCCCCGACGCCACCCTGCGGGAC
                    ************************************************************

Rv1819c             ACGCTGACCAAGGTGGCGCTGGCCCCACTGTGTGACCGGCTGGACGAGGAACGCGACTGG
Rv1819c_MT433       ACGCTGACCAAGGTGGCGCTGGCCCCACTGTGTGACCGGCTGGACGAGGAACGCGACTGG
                    ************************************************************

Rv1819c             GCCAAGGTGCTCTCCCCCGGTGAGCAGCAACGTGTTGCCTTTGCTCGCATCCTGCTCACC
Rv1819c_MT433       GCCAAGGTGCTCTCCCCCGGTGAGCAGCAACGTGTTGCCTTTGCTCGCATCCTGCTCACC
                    ************************************************************

Rv1819c             AAACCCAAGGCGGTCTTCCTCGACGAAAGTACCTCGGCGCTGGACACCGGGCTGGAGTTT
Rv1819c_MT433       AAACCCAAGGCGGTCTTCCTCGACGAAAGTACCTCGGCGCTGGACACCGGGCTGGAGTTT
                    ************************************************************

Rv1819c             GCGCTCTACCAATTGCTGCGCAGCGAGCTGCCGGACTGCATCGTGATCAGCGTCAGCCAT
Rv1819c_MT433       GCGCTCTACCAATTGCTGCGCAGCGAGCTGCCGGACTGCATCGTGATCAGCGTCAGCCAT
                    ************************************************************

Rv1819c             CGCCCCGCCCTCGAGCGGCTGCACGAAAACCAGCTCGAACTACTCGGTGGCGGCCAATGG
Rv1819c_MT433       CGCCCCGCCCTCGAGCGGCTGCACGAAAACCAGCTCGAACTACTCGGTGGCGGCCAATGG
                    ************************************************************

Rv1819c             CGGCTGGCCCCAGTCGAGGCGGCGCCCGCCGAAGTGTAAGGATCC--------------
Rv1819c_MT433       CGGCTGGCCCCAGTCGAGGCGGCGCCCGCCGAAGTGTAAGGATCCGCCGGGACGAGCTTC
                    *******************************************

Rv1819c             ------------------------------------------------------------
Rv1819c_MT433       CCAAATCGGCGTGGCCGCTTCCGTGATCGCGATACGCTGAATCCGTCGATCTCTGGGGAG
```

APPENDIX E (CONTINUED)

2. Nucleotide sequence of Rv1877 of *M. tuberculosis* MT433

```
Rv1877          ------------------------------------------------------------
Rv1877_MT433    ATTCTCCGATACCACTCCGGGCGCCGCTGACAAGCTCTAGCATCGACTCGAACAGCGATG

Rv1877          -----------GGATCATGGCGGGCCCCACAGCACCGACCACTGCCCCCACCGCAATCCG
Rv1877_MT433    GGAGGGCGGATGGATCATGGCGGGCCCCACAGCACCGACCACTGCCCCCACCGCAATCCG
                           **************************************************

Rv1877          AGCCGGTGGCCCGCTGCTCAGTCCGGTGCGACGCAACATTATTTTCACCGCACTTGTGTT
Rv1877_MT433    AGCCGGTGGCCCGCTGCTCAGTCCGGTGCGACGCAACATTATTTTCACCGCACTTGTGTT
                ************************************************************

Rv1877          CGGGGTGCTGGTCGCTGCGACCGGCCAAACCATCGTTGTGCCCGCATTGCCGACGATCGT
Rv1877_MT433    CGGGGTGCTGGTCGCTGCGACCGGCCAAACCATCGTTGTGCCCGCATTGCCGACGATCGT
                ************************************************************

Rv1877          CGCCGAGCTGGGCAGCACCGTTGACCAGTCGTGGGCGGTCACCAGCTATCTGCTGGGGGG
Rv1877_MT433    CGCCGAGCTGGGCAGCACCGTTGACCAGTCGTGGGCGGTCACCAGCTATCTGCTGGGGGG
                ************************************************************

Rv1877          AACTGTCGTGGTTGTGGTGGCTGGCAAGCTCGGTGATCTGCTCGGCCGCAACAGGGTGCT
Rv1877_MT433    AACTGTCGTGGTTGTGGTGGCTGGCAAGCTCGGTGATCTGCTCGGCCGCAACAGGGTGCT
                ************************************************************

Rv1877          GCTAGGCTCCGTCGTGGTCTTCGTCGTTGGCTCTGTGCTGTGCGGGTTATCGCAGACGAT
Rv1877_MT433    GCTAGGCTCCGTCGTGGTCTTCGTCGTTGGCTCTGTGCTGTGCGGGTTATCGCAGACGAT
                ************************************************************

Rv1877          GACCATGCTGGCGATCTCTCGCGCACTGCAGGGCGTCGGTGCCGGTGCGATTTCCGTCAC
Rv1877_MT433    GACCATGCTGGCGATCTCTCGCGCACTGCAGGGCGTCGGTGCCGGTGCGATTTCCGTCAC
                ************************************************************

Rv1877          CGCCTACGCGCTGGCCGCTGAGGTGGTCCCACTGCGGGACCGTGGCCGCTACCAGGGCGT
Rv1877_MT433    CGCCTACGCGCTGGCCGCTGAGGTGGTCCCACTGCGGGACCGTGGCCGCTACCAGGGCGT
                ************************************************************

Rv1877          CTTAGGTGCGGTGTTCGGTGTCAACACGGTCACCGGTCCGCTGCTGGGGGGCTGGCTCAC
Rv1877_MT433    CTTAGGTGCGGTGTTCGGTGTCAACACGGTCACCGGTCCGCTGCTGGGGGGCTGGCTCAC
                ************************************************************

Rv1877          CGACTATCTGAGCTGGCGGTGGGCGTTTTGGATCAACGTGCCGGTTTCGATCGCGGTGCT
Rv1877_MT433    CGACTATCTGAGCTGGCGGTGGGCGTTTTGGATCAACGTGCCGGTTTCGATCGCGGTGCT
                ************************************************************

Rv1877          GACAGTGGCGGCAACCGCCGTCCCTGCGTTGGCCCGACCGCCCAAACCGGTCATCGACTA
Rv1877_MT433    GACAGTGGCGGCAACCGCCGTCCCTGCGTTGGCCCGACCGCCCAAACCGGTCATCGACTA
                ************************************************************
```

APPENDIX E (CONTINUED)

2. Nucleotide sequence of Rv1877 of *M. tuberculosis* MT433

```
Rv1877          CCTTGGGATCCTGGTCATCGCTGTGGCCACGACCGCTTTGATCATGGCCACAAGTTGGGG
Rv1877_MT433    CCTTGGGATCCTGGTCATCGCTGTGGCCACGACCGCTTTGATCATGGCCACAAGTTGGGG
                ************************************************************

Rv1877          CGGAACCACCTACGCCTGGGGCTCAGCGACCATTGTCGGGCTGTTGATCGGGGCCGCAGT
Rv1877_MT433    CGGAACCACCTACGCCTGGGGCTCAGCGACCATTGTCGGGCTGTTGATCGGGGCCGCAGT
                ************************************************************

Rv1877          GGCGCTGGGTTTCTTCGTGTGGCTGGAGGGCCGCGCCGCTGCGGCCATCCTGCCGCCCAG
Rv1877_MT433    GGCGCTGGGTTTCTTCGTGTGGCTGGAGGGCCGCGCCGCTGCGGCCATCCTGCCGCCCAG
                ************************************************************

Rv1877          GCTGTTTGGCAGCCCAGTATTTGCCGTGTGCTGCGTCCTGTCCTTCGTGGTCGGATTCGC
Rv1877_MT433    GCTGTTTGGCAGCCCAGTATTTGCCGTGTGCTGCGTCCTGTCCTTCGTGGTCGGATTCGC
                ************************************************************

Rv1877          GATGCTGGGTGCACTGACCTTCGTACCGATCTATCTGGGGTACGTGGACGGCGCGTCGGC
Rv1877_MT433    GATGCTGGGTGCACTGACCTTCGTACCGATCTATCTGGGGTACGTGGACGGCGCGTCGGC
                ************************************************************

Rv1877          GACCGCGTCAGGTCTGCGCACGTTGCCGATGGTGATCGGCCTGCTGATCGCCTCGACCGG
Rv1877_MT433    GACCGCGTCAGGTCTGCGCACGTTGCCGATGGTGATCGGCCTGCTGATCGCCTCGACCGG
                ************************************************************

Rv1877          GACGGGTGTCCTGGTCGGCCGGACGGGCCGCTACAAGATCTTCCCGGTCGCGGGGATGGC
Rv1877_MT433    GACGGGTGTCCTGGTCGGCCGGACGGGCCGCTACAAGATCTTCCCGGTCGCGGGGATGGC
                ************************************************************

Rv1877          GCTGATGGCGGTTGCGTTCCTGCTGATGTCGCAGATGGACGAGTGGACGCCACCGCTGCT
Rv1877_MT433    GCTGATGGCGGTTGCGTTCCTGCTGATGTCGCAGATGGACGAGTGGACGCCACCGCTGCT
                ************************************************************

Rv1877          GCAATCGCTGTACCTGGTCGTCCTAGGTGCCGGCATCGGATTGTCCATGCAGGTGCTCGT
Rv1877_MT433    GCAATCGCTGTACCTGGTCGTCCTAGGTGCCGGCATCGGATTGTCCATGCAGGTGCTCGT
                ************************************************************

Rv1877          TCTCATCGTGCAGAACACGTCGTCTTTCGAAGACCTCGGCGTCGCAACATCGGGTGTGAC
Rv1877_MT433    TCTCATCGTGCAGAACACGTCGTCTTTCGAAGACCTCGGCGTCGCAACATCGGGTGTGAC
                ************************************************************

Rv1877          CTTCTTCCGGGTGGTCGGCGCCTCGTTTGGTACCGCAACATTCGGTGCGTTGTTCGTAAA
Rv1877_MT433    CTTCTTCCGGGTGGTCGGCGCCTCGTTTGGTACCGCAACATTCGGTGCGTTGTTCGTAAA
                ************************************************************

Rv1877          CTTCCTGGACCGAAGACTCGGTTCCGCGCTGACGTCGGGCGCCGTGCCTGTCCCGGCAGT
Rv1877_MT433    CTTCCTGGACCGAAGACTCGGTTCCGCGCTGACGTCGGGCGCCGTGCCTGTCCCGGCAGT
                ************************************************************

Rv1877          GCCATCTCCGGCTGTCTTGCATCAGCTGCCCCAGAGCATGGCCGCCCCGATCGTGCGGGC
Rv1877_MT433    GCCATCTCCGGCTGTCTTGCATCAGCTGCCCCAGAGCATGGCCGCCCCGATCGTGCGGGC
                ************************************************************
```

APPENDIX E (CONTINUED)

2. Nucleotide sequence of Rv1877 of *M. tuberculosis* MT433

```
Rv1877          ATATGCCGAGTCGCTCACCCAGGTGTTCCTTTGCGCGGTCTCGGTCACGGTGGTCGGTTT
Rv1877_MT433    ATATGCCGAGTCGCTCACCCAGGTGTTCCTTTGCGCGGTCTCGGTCACGGTGGTCGGTTT
                ************************************************************

Rv1877          CATCCTGGCGCTGTTGCTGCGAGAGGTACCGCTCACCGACATCCACGATGACGCCGACGA
Rv1877_MT433    CATCCTGGCGCTGTTGCTGCGAGAGGTACCGCTCACCGACATCCACGATGACGCCGACGA
                ************************************************************

Rv1877          CCTCGGCGACGGGTTCGGTGTGCCCAGAGCCGAATCGCCGGAGGATGTGTTGGAAATCGC
Rv1877_MT433    CCTCGGCGACGGGTTCGGTGTGCCCAGAGCCGAATCGCCGGAGGATGTGTTGGAAATCGC
                ************************************************************

Rv1877          GGTTCGGCGTATGCTGCCGAACGGGGTGCGACTGCGCGATATTGCGACACAACCCGGTTG
Rv1877_MT433    GGTTCGGCGTATGCTGCCGAACGGGGTGCGACTGCGCGATATTGCGACACAACCCGGTTG
                ************************************************************

Rv1877          CGGACTCGGCGTCGCCGAGCTGTGGGCCCTTCTGCGGATCTATCAATACCAGCGGCTGTT
Rv1877_MT433    CGGACTCGGCGTCGCCGAGCTGTGGGCCCTTCTGCGGATCTATCAATACCAGCGGCTGTT
                ************************************************************

Rv1877          CGAGGCAGTACGGCTGACCGATATCGGTAGACACCTGCACGTGCCCTATCAGGTCTTTGA
Rv1877_MT433    CGAGGCAGTACGGCTGACCGATATCGGTAGACACCTGCACGTGCCCTATCAGGTCTTTGA
                ************************************************************

Rv1877          ACCCGTCTTCGACCGTCTGGTCCAGACCGGCTACGCGGCACGCGACGGCGACATCTTGAC
Rv1877_MT433    ACCCGTCTTCGACCGTCTGGTCCAGACCGGCTACGCGGCACGCGACGGCGACATCTTGAC
                ************************************************************

Rv1877          GCTAACCCCGTCCGGGCACCGTCAGGTCGACTCCCTCGCAGTTTTGATCCGTCAGTGGCT
Rv1877_MT433    GCTAACCCCGTCCGGGCACCGTCAGGTCGACTCCCTCGCAGTTTTGATCCGTCAGTGGCT
                ************************************************************

Rv1877          GCTCGACCACTTGGCCGTGGCGCCCGGCTTGAAGCGACAGCCAGACCACCAATTCGAAGC
Rv1877_MT433    GCTCGACCACTTGGCCGTGGCGCCCGGCTTGAAGCGACAGCCAGACCACCAATTCGAAGC
                ************************************************************

Rv1877          CGCTCTGCAGCACGTCACCGACGCGGTGCTCGTTCAACGAGACTGGTATGAAGATCTGGG
Rv1877_MT433    CGCTCTGCAGCACGTCACCGACGCGGTGCTCGTTCAACGAGACTGGTATGAAGATCTGGG
                ************************************************************

Rv1877          CGACCTGTCGGAATCACGCCAACTCGCGGCTACAACGTAGGGATCC--------------
Rv1877_MT433    CGACCTGTCGGAATCACGCCAACTCGCGGCTACAACGTAGGGATCCCGATGCTTGCCGCG
                *******************************************

Rv1877          ------------------------------------------------------------
Rv1877_MT433    CGTAGCCGCGCGAGCTGATCCGCGCTGCAGAATGACTGCCATGACAGCCACACCGCTTGC
```

APPENDIX E (CONTINUED)

3. Nucleotide sequence of Rv2846c of *M. tuberculosis* MT433

```
Rv2846c           ------------------------------------------------------------
Rv2846c_MT433     TCCCGCGATCATCGTGATCGGGGCTGTAGTCGGCCTGAGCGGCGTTCGGGGTTTAAACAA


Rv2846c           ------------------------------GGATCCATGACGGCTCTCAACGACACAGAGC
Rv2846c_MT433     TTCTTAAGAATACTGTAAGGTAACCCGCTGGATCCATGACGGCTCTCAACGACACAGAGC
                                               *******************************

Rv2846c           GGGCGGTCCGTAACTGGACAGCCGGACGCCCACACCGTCCGGCCCCGATGCGCCCGCCGC
Rv2846c_MT433     GGGCGGTCCGTAACTGGACAGCCGGACGCCCACACCGTCCGGCCCCGATGCGCCCGCCGC
                  ************************************************************

Rv2846c           GCTCGGAGGAGACCGCTTCAGAGCGCCCCAGCAGGTACTACCCGACTTGGCTGCCCTCGC
Rv2846c_MT433     GCTCGGAGGAGACCGCTTCAGAGCGCCCCAGCAGGTACTACCCGACTTGGCTGCCCTCGC
                  ************************************************************

Rv2846c           GCAGCTTTATCGCTGCGGTTATTGCTATCGGCGGGATGCAGCTGCTGGCGACCATGGACA
Rv2846c_MT433     GCAGCTTTATCGCTGCGGTTATTGCTATCGGCGGGATGCAGCTGCTGGCGACCATGGACA
                  ************************************************************

Rv2846c           GCACCGTCGCCATCGTCGCGCTACCTAAGATTCAAAACGAGCTGAGCTTGTCTGATGCCG
Rv2846c_MT433     GCACCGTCGCCATCGTCGCGCTACCTAAGATTCAAAACGAGCTGAGCTTGTCTGATGCCG
                  ************************************************************

Rv2846c           GCCGCAGCTGGGTGATCACCGCCTACGTGCTGACCTTCGGCGGGCTGATGCTGCTCGGCG
Rv2846c_MT433     GCCGCAGCTGGGTGATCACCGCCTACGTGCTGACCTTCGGCGGGCTGATGCTGCTCGGCG
                  ************************************************************

Rv2846c           GCCGGCTTGGCGACACCATCGGGCGCAAACGCACCTTCATTGTTGGCGTTGCGCTATTCA
Rv2846c_MT433     GCCGGCTTGGCGACACCATCGGGCGCAAACGCACCTTCATTGTTGGCGTTGCGCTATTCA
                  ************************************************************

Rv2846c           CCATCTCGTCGGTGCTGTGCGCGGTCGCCTGGGACGAGGCGACGTTGGTGATCGCCCGGT
Rv2846c_MT433     CCATCTCGTCGGTGCTGTGCGCGGTCGCCTGGGACGAGGCGACGTTGGTGATCGCCCGGT
                  ************************************************************

Rv2846c           TGTCCCAGGGTGTGGGGTCGGCCATCGCATCTCCGACCGGTCTGGCGCTGGTGGCGACCA
Rv2846c_MT433     TGTCCCAGGGTGTGGGGTCGGCCATCGCATCTCCGACCGGTCTGGCGCTGGTGGCGACCA
                  ************************************************************

Rv2846c           CGTTCCCCAAGGGACCTGCCCGCAACGCCGCGACGGCGGTGTTCGCCGCGATGACCGCGA
Rv2846c_MT433     CGTTCCCCAAGGGACCTGCCCGCAACGCCGCGACGGCGGTGTTCGCCGCGATGACCGCGA
                  ************************************************************

Rv2846c           TCGGGTCGGTGATGGGGCTGGTGGTCGGCGGAGCACTGACCGAGGTGTCATGGCGGTGGG
Rv2846c_MT433     TCGGGTCGGTGATGGGGCTGGTGGTCGGCGGAGCACTGACCGAGGTGTCATGGCGGTGGG
                  ************************************************************

Rv2846c           CGTTCCTGGTGAACGTGCCGATCGGGCTGGTGATGATCTACCTGGCCCGCACCGCCCTAC
Rv2846c_MT433     CGTTCCTGGTGAACGTGCCGATCGGGCTGGTGATGATCTACCTGGCCCGCACCGCCCTAC
                  ************************************************************
```

APPENDIX E (CONTINUED)

3. Nucleotide sequence of Rv2846c of *M. tuberculosis* MT433

```
Rv2846c             GGGAAACCAACAAAGAACGGATGAAGCTCGACGCCACCGGGGCCATACTGGCCACGCTGG
Rv2846c_MT433       GGGAAACCAACAAAGAACGGATGAAGCTCGACGCCACCGGGGCCATACTGGCCACGCTGG
                    ************************************************************

Rv2846c             CATGCACCGCGGCGGTTTTCGCCTTCTCGATCGGTCCTGAAAAGGGCTGGATGTCAGGCA
Rv2846c_MT433       CATGCACCGCGGCGGTTTTCGCCTTCTCGATCGGTCCTGAAAAGGGCTGGATGTCAGGCA
                    ************************************************************

Rv2846c             TTACCATCGGTTCGGGCCTGGTGGCCTTGGCGGCCGCTGTCGCGTTTGTCATCGTGGAGC
Rv2846c_MT433       TTACCATCGGTTCGGGCCTGGTGGCCTTGGCGGCCGCTGTCGCGTTTGTCATCGTGGAGC
                    ************************************************************

Rv2846c             GCACTGCCGAGAACCCCGTCGTGCCGTTCCACTTGTTCCGCGACCGCAACCGGTTGGTCA
Rv2846c_MT433       GCACTGCCGAGAACCCCGTCGTGCCGTTCCACTTGTTCCGCGACCGCAACCGGTTGGTCA
                    ************************************************************

Rv2846c             CGTTCAGCGCGATCCTGTTGGCCGGCGGCGTCATGTTCAGCCTGACCGTCTGCATCGGCC
Rv2846c_MT433       CGTTCAGCGCGATCCTGTTGGCCGGCGGCGTCATGTTCAGCCTGACCGTCTGCATCGGCC
                    ************************************************************

Rv2846c             TGTACGTGCAGGACATCTTGGGCTACAGCGCGCTACGCGCGGGCGTAGGTTTCATCCCGT
Rv2846c_MT433       TGTACGTGCAGGACATCTTGGGCTACAGCGCGCTACGCGCGGGCGTAGGTTTCATCCCGT
                    ************************************************************

Rv2846c             TCGTCATCGCGATGGGAATCGGCCTAGGTGTGTCCTCGCAGCTGGTGTCCCGGTTTTCGC
Rv2846c_MT433       TCGTCATCGCGATGGGAATCGGCCTAGGTGTGTCCTCGCAGCTGGTGTCCCGGTTTTCGC
                    ************************************************************

Rv2846c             CACGGGTGTTGACCATCGGCGGCGGATATCTGCTATTCGGCGCCATGCTGTACGGCTCAT
Rv2846c_MT433       CACGGGTGTTGACCATCGGCGGCGGATATCTGCTATTCGGCGCCATGCTGTACGGCTCAT
                    ************************************************************

Rv2846c             TTTTCATGCACCGTGGTGTGCCCTACTTCCCCAACCTGGTCATGCCGATCGTCGTCGGCG
Rv2846c_MT433       TTTTCATGCACCGTGGTGTGCCCTACTTCCCCAACCTGGTCATGCCGATCGTCGTCGGCG
                    ************************************************************

Rv2846c             GGATTGGCATCGGCATGGCCGTCGTCCCGCTGACTCTGTCGGCGATCGCTGGCGTCGGCT
Rv2846c_MT433       GGATTGGCATCGGCATGGCCGTCGTCCCGCTGACTCTGTCGGCGATCGCTGGCGTCGGCT
                    ************************************************************

Rv2846c             TCGACCAGATCGGTCCGGTATCGGCAATTGCGCTGATGCTGCAGAGCCTGGGCGGTCCGC
Rv2846c_MT433       TCGACCAGATCGGTCCGGTATCGGCAATTGCGCTGATGCTGCAGAGCCTGGGCGGTCCGC
                    ************************************************************

Rv2846c             TGGTGCTCGCCGTCATCCAGGCTGTGATCACGTCGCGCACGCTGTACCTGGGCGGTACCA
Rv2846c_MT433       TGGTGCTCGCCGTCATCCAGGCTGTGATCACGTCGCGCACGCTGTACCTGGGCGGTACCA
                    ************************************************************

Rv2846c             CCGGTCCGGTGAAGTTCATGAACGACGTGCAGTTGGCCGCGCTTGACCACGCCTACACCT
Rv2846c_MT433       CCGGTCCGGTGAAGTTCATGAACGACGTGCAGTTGGCCGCGCTTGACCACGCCTACACCT
                    ************************************************************
```

APPENDIX E (CONTINUED)

3. Nucleotide sequence of Rv2846c of *M. tuberculosis* MT433

```
Rv2846c             ACGGCCTGCTGTGGGTGGCCGGAGCGGCCATCATCGTCGGCGGTATGGCGCTGTTTATCG
Rv2846c_MT433       ACGGCCTGCTGTGGGTGGCCGGAGCGGCCATCATCGTCGGCGGTATGGCGCTGTTTATCG
                    ************************************************************

Rv2846c             GGTATACGCCGCAGCAGGTTGCCCATGCGCAGGAGGTCAAGGAAGCGATCGACGCCGGCG
Rv2846c_MT433       GGTATACGCCGCAGCAGGTTGCCCATGCGCAGGAGGTCAAGGAAGCGATCGACGCCGGCG
                    ************************************************************

Rv2846c             AGCTGTAAGGATCC----------------------------------------------
Rv2846c_MT433       AGCTGTAAGGATCCCCCTTCCCTTGGCTGCATTGACGGGCACCATCGTTTTGCTGATGGC
                    **************
```

Author Biography

Name	Miss Angkanang Sowajassatakul
Date of Birth	28 February 1988
Address	138 M. 2, Nongtamlung Sub-district, Phantong District, Chonburi, 20160, Thailand
Education	2010 Bachelor of Science in Biotechnology GPA 3.45 King Mongkut's Institute of Technology Ladkrabang 2018 Doctor of Philosophy in Biotechnology GPA 3.83 King Mongkut's Institute of Technology Ladkrabang
Scholarship	Thailand Graduate Institute of Science and Technology Scholarship (TGIST, NSTDA)
Academic Publications :	1. Sowajassatakul, A. Prammananan, T. Chaiprasert, A. and Phunpruch, S. 2014. "Molecular characterization of amikacin, kanamycin and capreomycin resistance in M/XDR-TB strains isolated in Thailand" *BMC Microbiol.* 14 : 165. 2. Sowajassatakul, A. Olabisi, O. Prammananan, T. Chaiprasert, A. and Phunpruch, S. 2015. "Draft genome sequence of Amikacin- and kanamycin-resistant *Mycobacterium tuberculosis* MT433 without *rrs* and *eis* mutations" *Genome Announc.* 3 : 6.
Trainings:	1. Thailand's participant in Japan-East Asia Network of Exchange for Students and Youths Programme (JENESYS) for culture and technology exchange, 28 Jan - 2 Feb 2012. Tokyo and Sendia, Japan. 2. Workshop's participant in 8th International Young Scientists School "System Biology and Bioinformatics" (SBB) at Institute of Cytology and Genetics, 20 - 29 Aug 2016. Novosibrisk, Russia.