ผลของยีน *RELE* ของเชื้อวัณโรคต่อการเจริญของเชื้อ *MYCOBACTERIUM SMEGMATIS* ในสภาวะเครียด

EFFECT OF *MYCOBACTERIUM TUBERCULOSIS RELE* ON GROWTH OF *MYCOBACTERIUM SMEGMATIS* IN STRESSFUL CONDITIONS

วิชาณี แบนคีรี

WICHANEE BANKEEREE

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์

สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

พ.ศ.2552

KMITL-2009-SC-M-020-032

EFFECT OF *MYCOBACTERIUM TUBERCULOSIS RELE* ON GROWTH OF *MYCOBACTERIUM SMEGMATIS* IN STRESSFUL CONDITIONS

WICHANEE BANKEEREE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE IN BIOTECHNOLOGY

FACULTY OF SCIENCE

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

2009

KMITL-2009-SC-M-020-032

คณะวิทยาศาสตร์

สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

ใบรับรองวิทยานิพนธ์

หัวข้อวิทยานิพนธ์ ผลของยีน *relE* ของเชื้อวัณโรคต่อการเจริญของเชื้อ *Mycobacterium smegmatis* ในสภาวะเครียด

Effect of *Mycobacterium tuberculosis relE* on Growth of *Mycobacterium smegmatis* in Stressful Conditions

นักศึกษา นางสาววิชาณี แบนคีรี

รหัสประจำตัว 48068305

ปริญญา วิทยาศาสตรมหาบัณฑิต

สาขาวิชา เทคโนโลยีชีวภาพ

อาจารย์ที่ปรึกษาวิทยานิพนธ์ ผศ.ดร.สรัญญา พันธุ์พฤกษ์

อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม ดร.เทอดศักดิ์ พราหมณะนันทน์

คณะกรรมการสอบวิทยานิพนธ์		ลายมือชื่อ
ผศ.ดร.พนา	โลหะทรัพย์ทวี	
ผศ.ดร.สรัญญา	พันธุ์พฤกษ์	
ดร.เทอดศักดิ์	พราหมณะนันทน์	
รศ.ดวงใจ	โอชัยกุล	
ดร.วันดี	ยินดียั่งยืน	

สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

วัน / เดือน/ ปี ที่สอบ 19 พฤษภาคม 2552 **เวลา** 10.00 – 12.00 น.

สถานที่สอบ ณ อาคารจุฬาภรณวลัยลักษณ์ 1 ห้อง 424

คณะวิทยาศาสตร์รับรองแล้ว

(รองศาสตราจารย์ ดร.ธีรวัฒน์ มงคลอัศวรัตน์)

คณบดีคณะวิทยาศาสตร์

วันที่ 26 **เดือน** พฤษภาคม **พ.ศ.** 2552

Thesis Title	Effect of *Mycobacterium tuberculosis relE* on growth of *Mycobacterium smegmatis* in stressful conditions
Student	Miss Wichanee Bankeeree
Student ID.	48068305
Degree	Master degree of Science
Program	Biotechnology
Year	2009
Thesis Advisor	Asst. Prof. Dr. Saranya Phunpruch
Thesis Co-Advisor	Dr. Therdsak Prammananan

ABSTRACT

Tuberculosis (TB) is a bacterial infectious disease caused by *Mycobacterium tuberculosis*. TB is in the top ten cause of deaths of world's population and is in the first rank of death due to bacterial infection in Thailand. TB has been exacerbated by HIV epidemic and the emergence of multidrug-resistant tuberculosis (MDR-TB). Many efforts have been taken for fighting against this disease, including the development of new antituberculous drugs that could be effective against both MDR-TB and latent TB, and also shorten the time of treatment. One strategy for developing new drugs is searching for the potential targets, which are essential for cell survival and pathogenesis, particularly for the dormancy state. The regulatory system, like toxin-antitoxin (TA) module, has been investigated in many organisms, and shown to be important for bacteria. Interference the function of this system should impair the bacterial viability, especially in the presence of stresses. One of the TA modules, *relBE*, which has been shown to cause translational relax in bacteria in response to some stressful conditions and might play a role in *M. tuberculosis* for entering the dormancy state, was investigated in this study. In *M. tuberculosis*, at least three loci of *relBE* have been identified with unknown function. This study aims to investigate the role of *M. tuberculosis relE*, a toxin-like gene, on growth in response to various stressful conditions. The *M. tuberculosis relE* gene was cloned into the replicative mycobacterial shuttle vector, pMV261. The correct recombinant plasmid was transformed into *M. smegmatis* mc^2155. The effect of RelE on growth under stressful conditions like acid environment, oxidative stress, temperature stress and nutritional starvation was determined in the test strain, *M. smegmatis* mc^2155::pMV261-*relE*, compared to the control strain, *M. smegmatis* mc^2155::pMV261. Among

the various stressful conditions in this study, the results showed that the stressful conditions as exposure to reactive oxygen intermediate and cultivation under nutritional starvation showed significant difference ($p<0.05$) of growth or survival rate between control and test strain. No significant difference ($p>0.05$) between test and control strains was found under acid condition, exposure to reactive nitrogen intermediate and high temperature.

หัวข้อวิทยานิพนธ์	ผลของยีน *relE* ของเชื้อวัณโรคต่อการเจริญของเชื้อ *Mycobacterium smegmatis* ในสภาวะเครียด
นักศึกษา	นางสาววิชาณี แบนคีรี
รหัสนักศึกษา	48068305
ปริญญา	วิทยาศาสตรมหาบัณฑิต
สาขาวิชา	เทคโนโลยีชีวภาพ
พ.ศ.	2552
อาจารย์ที่ปรึกษา	ผศ.ดร.สรัญญา พันธุ์พฤกษ์
อาจารย์ที่ปรึกษาร่วม	ดร.เทอดศักดิ์ พราหมณะนันทน์

บทคัดย่อ

วัณโรคคือโรคติดเชื้อที่เกิดจากแบคทีเรีย *Mycobacterium tuberculosis* ที่ยังคงติดอยู่ในสาเหตุการตาย 10 อันดับแรกของประชากรโลกและเป็นสาเหตุการเสียชีวิตอันดับแรกของผู้ป่วยที่ติดเชื้อแบคทีเรียในประเทศไทย นอกจากนี้ ยังพบการเพิ่มจำนวนของผู้ป่วยติดเชื้อ HIV ร่วมกับเชื้อวัณโรคและผู้ป่วยวัณโรคดื้อยาในบางภูมิภาคของโลก ทำให้การควบคุมป้องกันวัณโรคทำได้ยากยิ่งขึ้น จึงเป็นความจำเป็นเร่งด่วนในการพัฒนายาต้านวัณโรคตัวใหม่ที่ออกฤทธิ์ได้ทั้งกับเชื้อวัณโรคดื้อยาหลายขนานและเชื้อวัณโรคในระยะแอบแฝง และสามารถลดระยะเวลาในการรักษาให้สั้นลง วิธีการหนึ่งที่ใช้ในการพัฒนายาใหม่ คือการค้นหาเป้าหมายของยาที่มีความจำเป็นต่อการอยู่รอดและการก่อโรคของเชื้อ มีรายงานการพบระบบการควบคุมยีนที่เรียกว่าทอกซิน-แอนติทอกซินในแบคทีเรียหลายชนิดและพบว่ามีความสำคัญกับแบคทีเรียโดยเฉพาะการอยู่รอดของเซลล์เพื่อตอบสนองต่อสภาวะเครียด หนึ่งในยีนเหล่านี้ ได้แก่ กลุ่มยีน *relBE* ที่ควบคุมการแปลรหัสพันธุกรรมของแบคทีเรียในสภาวะเครียดและมีอาจส่วนสำคัญในการเข้าสู่ระยะแอบแฝงของเชื้อวัณโรค ในเชื้อวัณโรคพบกลุ่มยีน *relBE* อย่างน้อยสามบริเวณ แต่ยังไม่ทราบหน้าที่แน่ชัด ดังนั้น งานวิจัยนี้จึงมีวัตถุประสงค์ในการศึกษาผลของยีนทอกซิน *relE* ต่อการเจริญในการตอบสนองต่อสภาวะเครียดต่างๆ โดยโคลนยีน *relE* ของเชื้อวัณโรค เข้ากับพลาสมิดแสดงออกของมัยโคแบคทีเรีย pMV261 รีคอมบิแนนท์พลาสมิดที่ถูกต้องถูกทรานสฟอร์มเข้าสู่เชื้อ *M. smegmatis* mc^2155 และนำไปศึกษาถึงผลการแสดงออกของยีน *relE* ในสภาวะเครียดต่างๆ เช่น สภาวะที่อาหารมีความเป็นกรด สภาวะที่มี ROI และ RNI สภาวะขาดสารอาหาร และสภาวะที่มีอุณหภูมิสูง เปรียบเทียบสายพันธุ์นี้ (*M. smegmatis* MC^2155::pMV261-*relE*) กับสายพันธุ์ดั้งเดิม (*M. smegmatis* MC^2155::pMV261) จากการศึกษาพบว่าสภาวะที่ส่งผลกระทบในเชิงลบต่อการเจริญของเชื้อ *M. smegmatis* mc^2155 ที่มียีน *relE* ของเชื้อวัณโรคได้แก่ สภาวะที่มี ROI และสภาวะ

ขาดสารอาหาร โดยเชื้อที่ทดสอบจะมีอัตราการอยู่รอดหรืออัตราการเจริญที่ลดลงอย่างเห็นได้ชัด โดยแตกต่างอย่างมีนัยสำคัญที่ระดับความเชื่อมั่นร้อยละ 95 กับเชื้อควบคุม ขณะที่สภาวะเครียดอื่นๆที่ทำการศึกษาไม่แสดงความแตกต่างอย่างมีนัยสำคัญระหว่างสายพันธุ์ที่ระดับความเชื่อมั่นร้อยละ 95

ACKNOWLEDGEMENT

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. First, I want to thank the Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA), TGIST 01–49-101, for supporting found to this study. Many thanks to my major professor, Asst. Prof. Dr. Saranya Phunpruch, who allowing me to enter her lab and perform research. Her sponsorship and mentorship have made it possible to reach my goal of obtaining an advanced degree. I thank her for the opportunity that she has afforded me. I also express my deepest gratitude to Dr. Therdsak Prammananan for critical review of the manuscript and for helpful suggestions and discussions. Additionally, I would also like to express my gratitude to my graduate committee members, Asst. Prof. Dr. Pana Lohasupthawee, Assoc. Prof. Duangjai Ochaikul and Dr. Wandee Yindeeyangyuen. I am extremely grateful for your guidance and constructive criticism. I want to thank my colleagues from the department of biotechnology, King Mongkut's Institute of Technology Ladkrabang (KMITL), for all their help, support, interest and valuable hints. Especially I am obliged to Miss Sirinya Jaitrong, Miss Rungarun Suksumran, Miss Surattiporn Rattana and Mr. Samart Taiklao. They have been very motivating during the inevitable low periods of graduate research. Last but perhaps the most significant, I appreciate the support of my parents, before and during graduate studies. I would have been nowhere without their sacrifices and enormous struggle for my education. I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally one by one.

Wichanee Bankeeree

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

Page

TABLE OF CONTENTS (CONTINUED)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is still a public health problem in Thailand and worldwide. More than 80% of all TB patients live in sub-Saharan Africa and Asia (WHO, 2006a). Overall 34% are in South-East Asia. Thailand, the 17th rank of countries with highest TB burden, reported approximately 90,000 new TB cases annually, of which 40,000 cases are active TB and 12,000 die of TB. Typically, treatment of tuberculosis relies on combination of four first-line antituberculous drugs, namely isoniazid, rifampicin, ethambutal and pyrazinamide. In contrast to infection with drug-susceptible *M. tuberculosis* strains, patients who are infected with drug-resistant strains need more drugs and time to eradicate the bacteria up to 24 months. However, with 6 month-period of treatment, it is easier to generate drug-resistant strains in patients who had incomplete treatment. The emergence of multidrug-resistant tuberculosis (MDR-TB) and also extensively drug-resistant tuberculosis (XDR-TB), which causes high mortality rate particularly in HIV-infected patients, makes the prevention and control of TB more difficult.

One of the most remarkable features of *M. tuberculosis* is its ability to switch to a latent infection. By slowing metabolism or becoming dormant, the bacteria may counterbalance these conditions and appear as silent to the immune system (Cardona and Ruiz-Manzano, 2004). Estimations suggest that, once infected; only 10% of the hosts will develop TB. It is believed that 5% of the infected population will develop the disease after 5 years and the others will suffer from it at some time during their lives (Chopra *et al.*, 2003). These make the urgent need to develop more effective and practical antituberculous drugs with an ideal that the new drugs could shorten the time of treatment and be effective against both MDR-TB and latent TB. However, basic knowledge on the pathogenesis of disease, particularly how the bacteria switch to the dormant state is very limited. After releasing the complete genome sequence of *M. tuberculosis* H37Rv, research on this issue has been intensively studied and facilitated the development of more rational and specific methods to search for new drug target.

One of the potential drug targets for new anti-TB drugs other than enzymes involved in the essential metabolic process within the cells is the regulatory proteins that response to different

stress conditions, such as starvation or growth under oxidative stress; disrupting or interferring the function of these proteins could affect their survival. The toxin-antitoxin module proposed in this study is also one of the unknown regulatory systems in mycobacteria. The mechanisms governing the switch to a persistent phenotype remain to be identified. However, accumulating evidence implicates the activity of the prokaryotic stringent response regulator, RelA that catalyzes the hyperphosphorylation of GTP to (p)ppGpp during amino acid and carbon source starvation. (p)ppGpp affects the global transcriptional response to changing environmental conditions by mediating association of alternative σ factors with RNA polymerase, in combination with toxin-antitoxin (TA) activity, and regulates a switch to a persistent phenotype or survival of *M. tuberculosis* during long-term starvation *in vitro* (Warner and Mizrahi, 2006). TA modules were originally characterized as factors ensuring episomal stability by post-segregational killing of cured segregants. The stable toxins inhibit transcription and translation by various mechanisms, including inhibition of DNA gyrase activity or cleavage of mRNA and require neutralization by labile antitoxins. Although subsequent analysis has revealed the widespread distribution of chromosomally encoded TA modules in prokaryotic genomes, *M. tuberculosis* also contains an unusually large number of putative TA loci in its genome such as *relBE* modules.

Prokaryotic chromosomes contain toxin-antitoxin loci, designated as "addiction modules", which are composed of two genes organized in an operon. One encodes a stable toxin and another encodes a labile cognate antitoxin (Gerdes, 2000). RelBE system is one of the toxin-antitoxin families that is conserved in various bacteria, including mycobacteria. *relB* and *relE* gene encode the metabolically labile antitoxin RelB and the stable toxin RelE, respectively. In steady state, antitoxins neutralize the effects of toxins by direct protein-protein interactions (Galvani *et al.*, 2001). In addition, antitoxins and toxin-antitoxin complexs bind to their promoters within their own operons and negatively regulate their own transcription (Gotfredsen and Gerdes, 1998). Upon induction by environmental stresses, like amino acids and carbon starvation, labile antitoxins are degraded by the Lon protease or the bacterial proteosome system, thereby leading to rapid growth arrest and cell death by effects of toxins (Christensen *et al.*, 2001). It was shown that a wide variety of stress resulted in overexpression of RelE toxin proteins caused cell growth arrest in *E. coli* (Susanne *et al.*, 2001). These observations are suggestive of possible involvement of the TA genes in entering the dormancy state of *M. tuberculosis*. It has been recently reported that RelE functions as a ribosome-dependent ribonuclease (RNase) and preferentially cleaves the

mRNA at stop codons (Pedersen *et al.*, 2003). Therefore, RelE is a global inhibitor of translation and is activated during nutritional stress. From our preliminary study, at least 3 putative *relBE* loci have been identified in the whole genome of *M. tuberculosis* H37Rv by computational tools. This result was in agreement with the most recent study (Gerdes *et al.*, 2005), which identified three *relBE* loci in the genome of *M. tuberculosis* H37Rv. However, the function of these genes or how importance of these genes is still undefined in mycobacteria.

1.2 Goal and objectives

This study aims to investigate the effect of *M. tuberculosis* H37Rv *relE* gene in growth response to various stressful conditions by using the *M. smegmatis* mc^2155 as an experimental model.

1.3 Scope of the study

1. Recombinant plasmids are constructed by amplifying *M. tuberculosis* H37Rv *relE* gene using polymerase chain reaction (PCR), subcloned into the TA cloning vector (pDrive, Qiagen, Germany), and transformed into *E. coli* DH5α.

2. The obtained transformants are analyzed for the presence of plasmid carrying the *relE* gene. DNA sequencing of the cloned *relE* gene is performed for determining the correct nucleotide sequence.

3. A replicative mycobacterial shuttle vector, pMV261, containing the *relE* gene is constructed and transformed into *M. smegmatis* mc^2155 in order to generate the *M. smegmatis* mc^2155 strain carrying the *M. tuberculosis relE* gene.

4. The effect of this toxin-like protein on growth of recombinant *M. smegmatis* mc^2155 under stressful conditions like acid environment, oxidative stress, temperature stress and nutritional starvation is determined compared to the parental strain.

1.4 Expected results

The role of *M. tuberculosis relE*, a toxin-like gene, in growth response to various stressful conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* complex such as *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti* and *Mycobacterium africanum*. It is an old disease but still ranks among the foremost killers of the 21st century. TB has been present in humans since antiquity. The earliest unambiguous detection of *M. tuberculosis* is in the remains of bison dated more over 18,000 years (Pearce-Duvet, 2006). Whether tuberculosis originated in cattle and then transferred to humans, or diverged from a common ancestor infecting different species, is currently unclear (Ernst *et al.*, 2007). However, it is clear that *M. tuberculosis* is not directly descended from *M. bovis*, which seems to have relatively evolved (Konomi *et al.*, 2002). In addition, genetic studies suggest that TB has been present in South America for about 2,000 years (Pearce-Duvet, 2006). According to the World Health Organization (WHO), over one-third of the world's population now has the TB bacteria in their bodies and new infections are occurring at rate of one per second (WHO, 2006b). Not everyone who is infected develops the disease and asymptomatic latent TB infection is most common. However, one in ten latent infections will progress to active TB disease which, if left untreated, kills more than half of its victims. In 2004, 14.6 million people had active TB and there were 8.9 million new cases and 1.7 million deaths, mostly in developing countries (NIAID, 2006). The South East Asian region accounted for the largest number of cases, 2.95 million, followed by the Western Pacific region, 1.9 million cases. Thailand is ranked 17th on the list of 22 high TB burden countries, an estimated 90,000 people develop TB annually giving an annual incidence of 206 TB cases per 100,000 people. HIV-associated TB now accounts for an estimated 15% of all TB cases in Thailand (Available: http://www.globalhealthreporting.org/countries/Thailand.asp). The global TB burden is increasing for several reasons such as poverty and widening gap between the rich and the poor, neglect of TB control, changing demography and the impact of the HIV pandemic (Raviglione and Luelmo, 1996). Drug-resistant strains of TB have emerged and are spreading. In 2000-2004, 20% of cases were resistant to standard treatment and 2% were also resistant to second-line drugs. TB incidence

varies widely and this appears to be caused by differences in healthcare. Thus, TB remains a major public health problem in most nations (Cole *et al.*, 1998).

2.1.1 Microbiology of mycobacteria

Mycobacteria are non-motile, pleomorphic rods and related to the Actinomyces. Most mycobacteria are found in habitats such as water, soil, cattle and birds or as normal pharyngeal flora in humans. There are about 70-80 species of mycobacteria, known as Nontuberculous mycobacteria (NTM), that cause neither TB nor leprosy (Stephen and Robert, 2006). However, a few are intracellular pathogens of animals and humans. *M. tuberculosis*, along with *M. bovis*, *M. africanum*, *M. canetti* and *M. microti* all cause the disease known as TB. *M. tuberculosis* is pathogenic for humans while *M. bovis* is usually pathogenic for bovine. *M. tuberculosis*, known as the tubercle bacillus, was first described on March 24, 1882 by Robert Koch, who subsequently received the Nobel Prize in physiology and medicine for this discovery in 1905. The bacterium is also known as Koch's bacillus (Camus *et al.*, 2002). *M. tuberculosis* was the cause of the "White Plague" of the 17th and 18th centuries in Europe. During this period nearly 100% of the European population was infected with *M. tuberculosis*, and 25% of all adult deaths were caused by *M. tuberculosis*. *M. tuberculosis* is an obligate aerobe. For this reason, in the classic case of TB, *M. tuberculosis* is always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence. It is a small bacillus that can withstand weak disinfectants and can survive in a dry state for weeks. Two media used to grow *M. tuberculosis* are Middlebrook medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. *M. tuberculosis* colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing *M. tuberculosis*. It takes 4-6 weeks to see colonies on both types of media. Fig. 2.1 shows small colonies of *M. tuberculosis* on Lowenstein-Jensen medium.

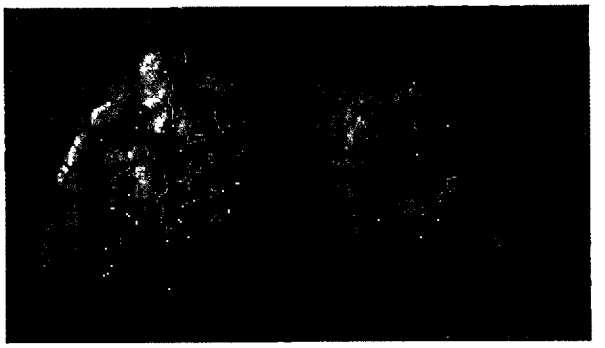


Figure 2.1 Colonies of *M. tuberculosis* on Lowenstein-Jensen medium
Available: http://en.wikipedia.org/wiki/Image:TB_Culture.jpg

M. tuberculosis is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either type, although *M. tuberculosis* contains peptidoglycan in its cell wall. If a Gram stain is performed on *M. tuberculosis*, it stains very weakly Gram-positive or not at all. Thus, *M. tuberculosis* is classified as acid-fast bacteria due to its impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and decolorized with acidified organic compounds. One acid-fast staining method for *M. tuberculosis*, Ziehl-Neelsen staining, shows red colonies of *M. tuberculosis* (Fig. 2.2).

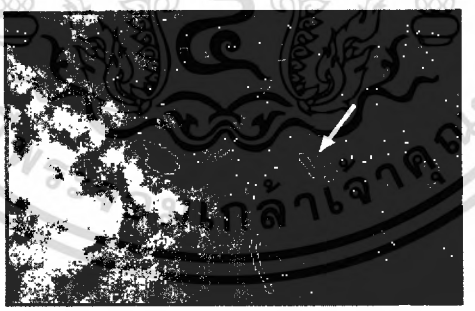


Figure 2.2 Red colonies of *M. tuberculosis* by Ziehl-Neelsen staining
Available: http://depts.washington.edu/hivaids/oit/case5/fig8d.html

The cell wall structure of *M. tuberculosis* deserves special attention because it is unique among prokaryotes, and it is a major determinant of virulence for this bacterium. The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of *M. tuberculosis*'s cell wall consists of three major components, mycolic acids (MA), cord factor, and wax-D. Structure of the mycobacterial

cell envelope was first proposed by Minnikin (1982) as shown in Fig. 2.3. The plasma membrane (PM) is shown at the bottom of the figure and is drawn with anchored lipomannan (LM), lipoarabinomannan (LAM) and a few representative proteins. The cell wall core components, peptidoglycan (PG), arabinogalactan (AG) and MA, are connected to each other. Cord factor is responsible for the serpentine cording. MAs are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface and thought to be a significant determinant of virulence in *M. tuberculosis*. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum. Cord factor is toxic to mammalian cells and most abundantly produced in virulent strains of *M. tuberculosis*. LAM is shown intercalated with both the PM and the capsule-like layer (CL). The CL is depicted containing LAM extended from the mycolates, arabinomannan (AM), glucan (GLU) and protein (Rastogi, 1991). The high concentration of lipids in the cell wall of *M. tuberculosis* has been associated with these properties of the mycobacterium such as impermeability to stains and dyes, resistance to many antibiotics, resistance to killing by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition and resistance to lethal oxidation and survival inside of macrophages (Barry *et al*., 1998).

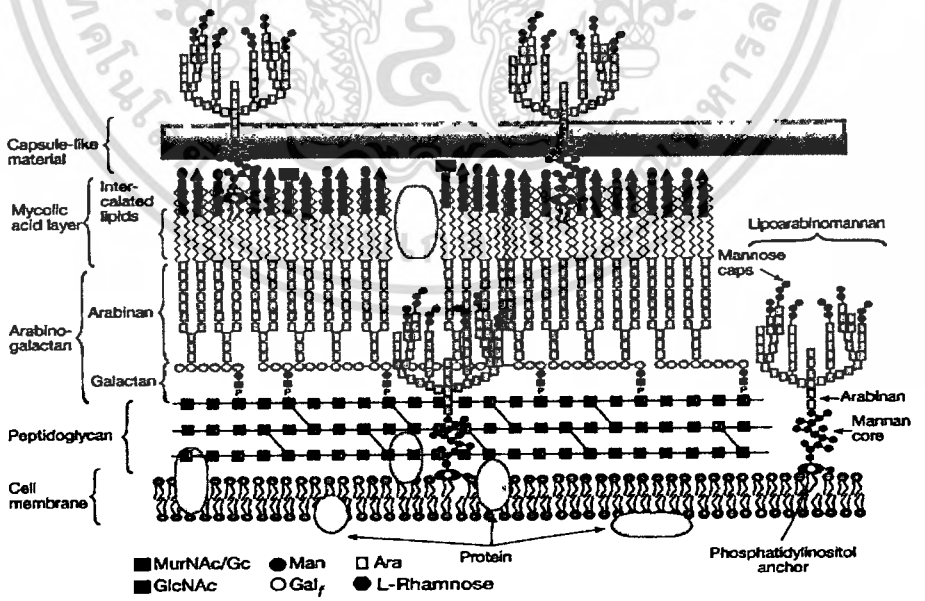


Figure 2.3 Structure of the cell wall of *M. tuberculosis*

Available : http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi/book=glyco2&part=ch20

The genome of the *M. tuberculosis* strain H37Rv was published in 1998 (Cole *et al.*, 1998). It size is 4 million base pairs, with 3,959 genes. Forty percent of these genes have had their function characterised, with possible function postulated for another 44%. Within the genome 6 pseudogenes are also included. The genome contains 250 genes involved in fatty acid metabolism, of which 39 genes involved in the polyketide metabolism generating the waxy coat. Such large numbers of conserved genes shows the evolutionary importance of the waxy coat to pathogen survival. Ten percent of the coding capacity is taken up by 2 cluster gene families that encode acidic glycine rich proteins. These proteins have a conserved N-terminal motif, deletion of which impairs growth in macrophages and granulomas (Glickman, 1994).

2.1.2 Pathogenesis

About 90% of *M. tuberculosis* infections are asymptomatic, latent TB infection, with only a 10% lifetime chance that a latent infection will progess to TB disease (Onyebujoh and Graham, 2006). The TB infection way and disease development in the body are determined by the risk of exposure to *M. tuberculosis*, the virulence of the invading organism and the innate and acquired defense mechanisms of the host (Rieder, 1999). *M. tuberculosis* does not produce toxin, but is able to destroy tissues through facultative intracellular multiplication. TB is spread by aerosol droplets expelled by people with the active disease of the lungs when they cough, sneeze, speak or kiss. These infectious droplets are 0.5 to 5 micrometers in diameter and about 40,000 droplets can be produced by a single sneeze. TB infection begins when the mycobacteria reach the pulmonary alveoli. The primary site of infection in the lungs is called the Ghon focus. Bacteria are picked up by dendritic cells and are transported to local lymph nodes. Further spread is through the bloodstream to the more distant tissues and organs where secondary TB lesions can develop in lung apices, peripheral lymph nodes, kidneys, brain and bone (Herrmann and Lagrange, 2005). All part of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid (Agarwal *et al.*, 2005).

TB is classified as one of the granulomatous inflammatory condition. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregrate to form granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only prevent dissemination of the mycobacteria, but also provide a local environment for cell communication of the immune system. Within granuloma, T lymphocytes (CD4+) secrete cytokines such as interferon gramma, which activates macrophages to destroy the bacteria which they are infected and T lymphocytes (CD8+) can also directly kill infected cells (Houben *et al.*, 2006).

The recognition and phagocytosis of mycobacteria can occur via mannan receptors or receptors for activation products of the complement system, including complement receptor 1 (CR1) as shown in Fig. 2.4. After being phagocytosed, the bacilli are processed into phagolysosomes, and the antigens (Ags) are presented to CD4+ T lymphocytes via major histocompatibility complex (MHC) class II cells. The fusion of phagosomes with endoplasmic reticulum or phagocytosed apoptotic vesicles can favor the presentation of Ags to CD8+ T cells via MHC class I. However, the activation of Toll-like receptors (TLRs) promotes the degradation and release of nuclear factor kappa B (NF-KB) which moves toward the cell nucleus and induces the activation of the transcription of a variety of genes that lead to the production of cytokines such as interleukin (IL)-12 and tumor necrosis factor alpha (TNF-α), as well as to the expression of co-stimulating molecules such as CD80 and CD86 (which interact with CD28). IL-23, IL-18 and IL-27 are also produced by the macrophages and, together with IL-12, they induce the production of interferon-gamma (IFN-γ) by T lymphocytes. The production of IL-2 and IL-2 receptors occurs in activated T cells and it induces the proliferation of T lymphocytes. IFN-γ and TNF-α activate macrophage microbicidal mechanisms. IFN-γ is also involved in the production of chemokines. IL-10, produced by macrophages and by T lymphocytes, acts as an endogenous immunosuppressant; TCR: T-cell receptor (T lymphocyte receptor); FcR: receptor for the Fc portion of antibodies (Ottenhoff *et al.*, 2005).

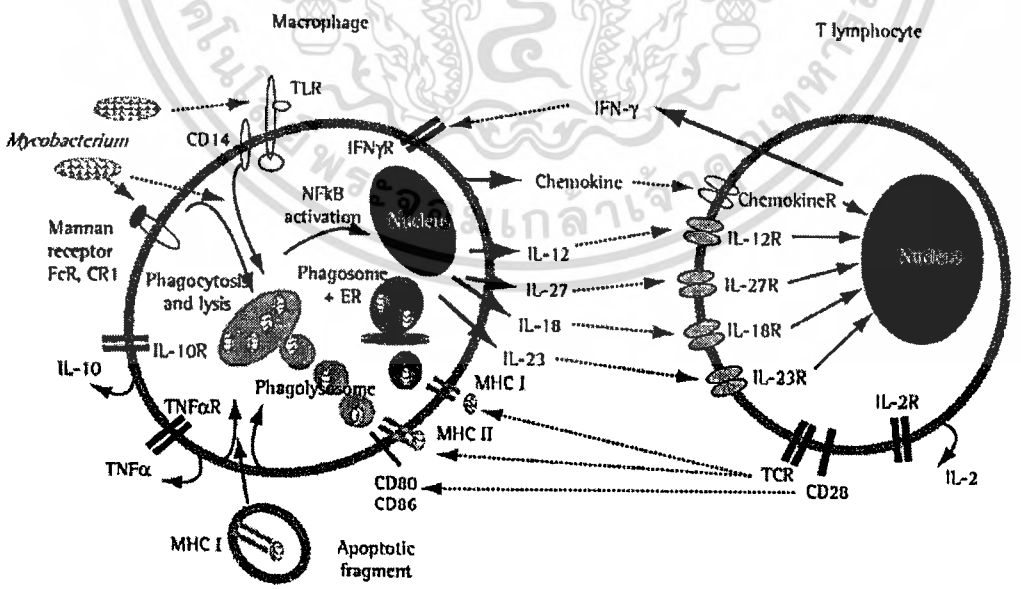


Figure 2.4 Mechanisms involved in the activation of macrophages and T lymphocytes by *M. tuberculosis*

Source: Teixeira *et al.* (2007)

Although the CD4+ T lymphocytes dominated immune response controls *M. tuberculosis* infection in the majority of otherwise healthy individuals, it does not completely eradicate infection. A small number of *M. tuberculosis* survives inside host macrophages, evading immune responses. The ability of *M. tuberculosis* 19-kD lipoprotein to inhibit MHC expression and Ags presentation may contribute to evasion of immune surveillance via a mechanism that is dependent on Toll-like receptor 2 (TLR2). A dependent-TLR2 inhibits MHC class II expression, preventing presentation of *M. tuberculosis* Ags and decreasing the recognition by T cells during later phases of macrophage infection. This mechanism may allow intracellular *M. tuberculosis* to evade immune surveillance and maintain chronic infection (Noss *et al.*, 2001). However, this mechanism requires hours to days to establish inhibition and cannot alter Ag processing during initial stages of macrophage infection. Other mechanisms, the ability of *M. tuberculosis* to inhibit phagosome maturation, may apply early after phagocytosis to enhance survival of *M. tuberculosis* and inhibit Ag processing. *M. tuberculosis* inhibits phagosomal maturation, decreasing phagosomal acidification, fusion of phagosomes with lysosomes, and phagosomal acquisition of lysosomal markers and characteristics. Thus, phagosomes containing live mycobacteria stain intensely for markers of early endosomes or immature phagosomes but only weakly for markers of late endosomes, lysosomes, and mature phagolysosomes. Loss of maturation is also associated with phagosome retention of tryptophan aspartate–containing coat (TACO), a coat protein of unknown function. Moreover *M. tuberculosis* was able to escape from fused phagosomes into vacuoles with membranes tightly opposed to the mycobacteria, and multiply. Mycobacteria are capable of producing ammonia, which could both inhibit phagosome-lysosome fusion and, by alkalinizing the intralysosomal contents, diminishing the potency of the fusion complex. Similarly, sulfatides, a glycolipid produced by *M. tuberculosis*, had been previously shown to also inhibit phagosome-lysosome fusion (Schluger and Rom, 1998).

Another feature of the granulomas of human TB is the development of cell death, also called necrosis, in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous necrosis (Kim *et al.*, 2003). *M. tuberculosis* gains entry to the bloodstream from an area of damaged tissue, it can spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues. This severe form of TB disease is most common in infants and the elderly and is called miliary tuberculosis. Patients with this disseminated TB have a fatality rate of approximately 20%, even with intensive treatment (Rothel and Andersen, 2005). Tissue destruction is balanced by healing and fibrosis (Parrish *et*

al., 1998). Affected tissue is replaced by scarring and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are joined to the air passages and this material can be coughed up. It contains living bacteria and can therefore cause infection. Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue (Niemann *et al.*, 2002).

Progression from TB infection to TB disease occurs when the TB bacilli overcome the immune system defenses and begin to multiply. In primary TB disease, it occurs soon after infection. However, in the majority of cases, a latent infection occurs that has no obvious symptoms. These dormant bacilli can produce TB in 2% to 23% of these latent cases, often many years after infection. The risk of reactivation increses with immunosuppression caused by infection with HIV. Some drugs, including rheumatoid arthritis drugs that work by blocking tumor necrosis factor-alpha, raise the risk of activating a latent infection due to the importance of this cytokine in immune defense against TB (Nahid *et al.*, 2006).

2.1.3 Symptoms

In the patients where TB becomes an active disease, 75% of these cases affect the lungs, where the disease is called pulmonary TB. Sympthoms include a productive, prolonged cough of more than three weeks duration, chest pain and coughing up blood. Systematic symptoms include fever, chills, night sweats, appetite loss, weight loss and paling, and those afflicted are often easily fatigued. When the infection spreads out of lungs, extrapulmonary sites include the pleura, central nervous system in meningitis, lymphatic system in scrofula of the neck, geniturinary system in urogenitial tuberculosis. An especially serious form is disseminated, or miliary tuberculosis. Extrapulmonary form is common in immunosuppressed persons and in young children. Infectious pulmonary TB may co-exist with extrapulmonary TB, which is not contagious.

2.1.4 Diagnosis

TB can be a difficult disease to diagnose, due mainly to the difficulty in culturing this slow-growing organism in the laboratory. A complete medical evaluation for TB must include a medical history, a chest X-ray, and a physical examination. Tuberculosis radiology is used in the diagnosis of TB. It may also include laboratory methods such as a tuberculin skin test, a serological test, microbiological smears, cultures and polymerase chain reaction detection of bacterial DNA (Pai *et al.*, 2008). Standard methods of laboratory confirmation of TB were divided as:

1. Tuberculin Skin Test (TST) is widely used as a supportive second line test to identify patients actively infected with tuberculosis. There are three types of tuberculin skin test but the most common is the Mantoux test. The skin test is done by injecting purified protein derivative (PPD) into the skin. PPD is a collection of mixed proteins and other materials filtered from killed MTB cultures. The test works on the basis that if the body has been exposed to infection with TB, it will recognize the proteins and mount an immune response to it. This response would take the form of a lump, swelling or blister at the site of injection. If there is a lump, called an induration, then this may mean that the person is infected. Unfortunately, the skin test has a poor sensitivity. This sensitivity falls further if the person being tested has had the BCG vaccination earlier in life or if they have a depressed immune system, immunocompromised, due to other illness or medical treatment.

2. Sputum Smear Microscopy (SSM) is the examination of sputum, matter thrown up from the lungs, for the detection of a certain type of bacteria. It is cheap and is performed in minutes. This test is based on the principle of Ziehl Neelsen diagnostic technique of direct smear microscopy of sputum. The unique properties of bacterial cell wall of MTB allow it to retain the primary stain even after exposure to strong acid solutions, they are called acid-fast. In the Ziehl Neelsen staining procedure, using carbol fuschsin and methylene blue, the acid-fast organisms appear red.

3. Cell culture techniques are still seen as the gold standard for active TB as they are extremely sensitive if live mycobacteria can be obtained in the sample. MTB can be cultured from a variety of specimens and can be used to detect pulmonary as well as extra-pulmonary disease. By assessing the effect of antibiotics on the cultured bacteria, this technique can also provide data on likely effectiveness of certain antibiotics. However, it is not always possible to obtain bacteria in the sample, especially in non-pulmonary TB and the test is therefore not always reliable. A drawback of this test is the time to result, which can be anything from two to six weeks.

4. Polymerase chain reaction (PCR) technique detects the presence of genetic material from bacteria by effectively amplifying the measurable amount. PCR is a relatively new development in active TB testing. Even though PCR techniques can magnify even the smallest amounts of genetic material, the sample used still has to contain a certain number of TB bacteria and this is not always possible, particularly with non-pulmonary TB where sensitivity can be as low as 60%. To increase the number of bacteria, and hence improve the sensitivity of

the test, the laboratory will often culture the sample, to allow the bacteria to multiply, before carrying out the PCR test. This can take several days or weeks. The test is also relatively complicated to run in the laboratory, is prone to cross contamination and can be expensive. The main use of PCR is not to diagnose TB, but to rule out other types of infection in a sputum smear positive patient, before culture results are known (http://www.tbdots.com/site/en/doctor_ section_ tb_diagnosis.html).

2.1.5 Treatment

Treatment for TB depends on whether a person has active TB or latent TB infection. A person who has latent TB might be given preventive therapy. Preventive therapy aims to kill TB bacteria that are currently inactive to prevent them from causing active TB disease in the future. First-line drugs for the treatment of TB consist of isoniazid (INH, H), rifampin (RMP, R), ethambutol (EMB, E) and pyrazinamide (PZA, Z). Latent TB infection is first treated with INH. The recommended length of treatment is nine months for children. Shorter courses of treatment with other first-line drugs may be used for special circumstances, such as exposure to someone who has TB that is resistant to INH. Patients with active TB commonly receive a combination of several drugs. Before RMP was available, TB patients had to take medication for 18-24 months. The combination of INH and RMP allowed completion of therapy within 9 months. Routine addition of PZA during the first two months has shortened duration of therapy to 6 months for most cases. EMB is known as a companion drug and has bacteriostatic activity. Its primary purpose is to suppress the further development of resistance in situations where INH resistance is already present at diagnosis. EMB can be discontinued as soon as the organism is known to be susceptible to both INH and RIF (Marra *et al.*, 2007).

However, these treatments are more difficult than the short courses of antibiotics used to cure most bacterial infections as long periods of treatment are needed to entirely eliminate mycobacteria from the body. It is very important that patients correctly take their medicine for the full length of treatment. If the medicine is incorrectly taken or treatment is stopped, the patient might become sick again and will be able to infect others with TB. In addition, if the treatment is not completed, the TB bacteria might become resistant to the medications and patients may develop multi-drug resistant TB. Treatment of active TB is challenging because patients must take multiple medications for at least 6 months. Directly observed therapy (DOT) is recommended by several international organizations for the standard regimen of TB treatment. In DOT, a health care trained worker monitors the patient taking each dose of anti-tuberculosis medication. DOT

was assigned based on a history of homelessness, illicit drug use, HIV infection, alcohol abuse, incarceration, age younger than 21 years, or inability to self-administer therapy. Treatment with properly implemented DOT has a success rate exceeding 95% and prevents the emergence of further multi-drug resistant strains of TB (http://www.in.gov/isdh/19686.htm). The first-line drugs are not interchangeable. Second-line drugs must be used when patients cannot take first-line drugs because of resistance or intolerance. These second-line agents are substantially less active, and not without risks of toxicity. Patients taking second-line drugs in lieu of both INH and RIF require treatment durations of up to 2 years with frequent monitoring for side effects. Second-line drugs are divided into 6 classes as aminoglycosides other than streptomycin (e.g., kanamycin and amikacin), cyclic polypeptides (e.g., capreomycin), fluoroquinolones (e.g., ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin), thioamides (e.g., prothionamide and ethionamide), serine analogs (e.g., cycloserine and terizidone), and salicylic acid derivatives (e.g., para-aminosalicyclic acid).

The treatment of tuberculosis in people infected with HIV requires close monitoring. It is especially important for those who are co-infected with HIV and TB to discuss TB treatment options with a health care worker to avoid potential complications, because some commonly prescribed medications to treat TB can interact with some antiretroviral drugs. The standard treatment regimen for TB patients who previously have been treated for the disease also may differ. Re-treatment cases also should be closely monitored because they have a higher likelihood of drug resistance, making treatment more difficult.

2.1.6 Drug-resistant tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to at least INH and RIF and has been reported from different regions of the world since the 1990s. It is the most severe form of bacterial resistance today and an important cause for concern in tuberculosis control (Zager and McNerney, 2008). The World Health Organization (WHO) in April 1993 declared tuberculosis a "Global Emergency" (WHO, 1992). To cure MDR-TB, health care providers must turn to a combination of second-line drugs. Second line drugs may have more side effects, the treatment may last much longer, and the cost may be up to 100 times more than first-line therapy. MDR-TB strains can also grow resistant to second-line drugs, further complicating treatment. Drug resistant tuberculosis is transmitted in the same way as regular TB. Primary resistance occurs in persons who are infected with a resistant strain of TB. A patient with fully-susceptible TB develops secondary resistance during TB therapy because of inadequate treatment,

not taking the prescribed regimen appropriately, or using low quality medication. Drug-resistant TB is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs (Mitnick *et al.*, 2003).

Extensively drug-resistant TB (XDR-TB) is defined as resistance to at least rifampicin, isoniazid, a second line injectable drug (capreomycin, kanamycin or amikacin) and a fluoroquinolone. The description of XDR-TB was first used earlier in 2006, following a joint survey by WHO and the US Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/tb/xdrtb/overview.htm). Resistance to anti-TB drugs in populations is a phenomenon that primarily occurs due to poorly managed TB care. Problems include incorrect drug prescribing practices by providers, poor quality drugs or erratic supply of drugs, and also patient non-adherence. Recent findings from a survey conducted by WHO and CDC on data from 2000-2004 found that XDR-TB has been identified in all regions of the world but is most frequent in the countries of the former Soviet Union and in Asia. Separate data on a recent outbreak of XDR-TB in an HIV-positive population in Kwazulu-Natal in South Africa was characterized by alarming high mortality rates. Scarce drug resistance data available from Africa indicated that while population prevalence of drug resistant TB appeared to be low compared to Eastern Europe and Asia, drug resistance in the region was on the rise. Given the underlying HIV epidemic, drug-resistant TB could have a severe impact on mortality in Africa and requires urgent preventative action. XDR-TB poses a grave public health threat, especially in populations with high rates of HIV and where there are few health care resources (Singh *et al.*, 2007).

Within the last 10 years, the mechanism of action of most antituberculosis agents has been described, same as the molecular mechanisms whereby *M. tuberculosis* becomes resistant. Resistance of MTB can occur through chromosomal mutation although rarely movement of mobile genetic elements, such as the insertion sequence IS*6110*, has been associated with new resistance emerging through the inactivation of critical genes. The different genes that have been associated with resistance to antituberculosis agents are summarized in Table 2.1. The genetic basis of resistance for some antituberculosis agents is not fully known. For example, streptomycin resistance emerges through mutations in *rrs* and *rpsL* that produce an alteration in the streptomycin binding site, but these changes are identified in just over one-half of the strains studied. Thus there is a considerable amount of research into the mechanisms of resistance that is still required. It should be noted that in many cases mutations found in association with drug-resistant organisms may cause different levels of resistance and also may not be directly related to

the mechanism of resistance. INH-resistance is a case in point. Modification of KatG, partial or total deletion, point mutation, or insertion, leads to the abolition or diminution of catalase activity and high-level resistance to INH. Catalase activity is essential in activating INH to the active hydrazine derivative. A deficiency in enzyme activity produces high-level resistance and is found in more than 80% of INH-resistant strains. Alternatively, low-level resistance can be caused by point mutation in the regulatory region of *inhA* operon, resulting in overexpression of *inhA*. Strains with this mutation have normal mycolic acid synthesis but low-level resistance to INH. Point mutation in the regulatory region of *ahpC* have also been demonstrated, these are a compensation for the effects of absent or reduced catalase (KatG) function and do not directly result in resistance. Most PZA-resistant organisms have mutations in the pyrazinamidase gene, although the gene may also be inactivated through the insertion of IS*6110*. PZA is essential in producing the active pyrazinoic acid derivative, and mutants are unable to produce an active drug (Gillespie, 2002).

Table 2.1 Summary of the gene involved in various kinds of antituberculosis drugs

Drug	Associated mutated gene or mutation
Rifampin	*rpoB*
Isoniazid	*katC, inhA, oxyR, ahpC, furA*
Streptomycin	*rrs, rpsL*
Pyrazinamide	*pncA*, IS*6110* insertion
Ethambutol	*embB*
Fluoroquinolone	*gyrA, gyrB*

Source: Gillespie, 2002

2.1.7 Vaccines

BCG vaccines are live vaccines derived from a strain of *M. bovis* that was attenuated by Calmette and Guérin at the Pasteur Institute in France between 1905 and 1921 (CDC, 2006). However, mass vaccination with BCG did not start until after World War II. Many countries use BCG vaccine as part of their TB control programs, especially for infants. The protective efficacy of BCG for preventing serious forms of TB in children is greater than 80%, it protects efficacy for preventing pulmonary TB in adolescents and adults is variable, ranging from 0% to 80% (John *et al.*, 1996). In South Africa, the country with the highest prevalence of TB, BCG is given to all children under the age of three (Strachan *et al.*, 1995). However, the effectiveness of BCG is lower in some areas where mycobacteria are less prevalent, therefore

BCG is not given to the entire populations in these countries. BCG vaccination is not recommended as a routine strategy for TB control in the United States. The use of BCG vaccine has been limited because its effectiveness in preventing infectious forms of TB is uncertain and the reactivity to tuberculin that occurs after vaccination interferes with the management of persons who are possibly infected with *M. tuberculosis*.

Several new vaccines to prevent TB infection are being developed. The first recombinant tuberculosis vaccine entered clinical trials in the United States in 2004, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) (Fine *et al.*, 2001). In 2005, it was shown that a DNA TB vaccine given with conventional chemotherapy can accelerate the disappearance of bacteria as well as protect against re-infection in mice, it may take four to five years to be available in humans (Martin, 2006). In 2008, vaccination with a recombinant modified vaccinia Ankara expressing antigen 85A from *M. tuberculosis*, MVA85A, induces high levels of cellular immune responses in volunteers. It is based on the striking effect of non-replicating poxviruses on the amplification of pre-existing T cell responses. Expressing a major secreted antigen of *M. tuberculosis*, antigen 85A, in the non-replicating modified vaccinia viral vector (MVA) strongly boosts BCG primed T cell responses in several species, including humans. Importantly, BCG-MVA85A prime-boost regimens have greater protective efficacy than BCG alone in several animal models of TB, including non-human primates (Brookes *et al.*, 2008).

2.2 Programmed cell death in bacteria

Programmed cell death (PCD) refers to death of cells mediated by an intracellular death program. PCD plays an important rule in number of developmental process in bacteria that contributes to maintenance of low mutation rate or mediates efficient plasmid stabilization by killing of plasmid free cells. The killing effect is called post-segregational killing (PSK) (Franch *et al.*, 1997). PSK is triggered by the unique genetic system that consists of a pair of genes such as toxin-antitoxin (TA) systems (Faridani *et al.*, 2006). Two types of TA systems that mediate PCD have been identified. In one type, the regulators are unstable antisense RNAs that inhibit the translation of stable, toxin-encoding mRNAs. The other type relies on stable toxins whose action is prevented by cognate protein antibody (Zielenkiewicz and Ceglowski, 2001).

2.2.1 Antisense RNA-regulated cell killing

Plasmid stabilization systems regulated by antisense RNA constitute a well conserved group called the *hok-sok* family. This family has been found only in Gram-negative

bacteria (Moller *et al.*, 2001). The *hok-sok* systems of plasmid R1 form *E. coli* increase plasmid maintenance through PSK of plasmid free cell. The *hok-sok* locus codes for three genes, *hok* (host killing) encodes a highly toxin *trans*-membrane protein that irreversibly damages the cell membrane (Thisted *et al.*, 1994a). The *mok* (modulation of killing) reading frame overlaps with *hok* sequence and is required for *hok* expression and translation as Fig. 2.5. Finally, the *sok* (suppression of killing) gene encodes a small antisense RNA in *cis* that blocks translation of the *mok* reading frame and inhibits expression of *hok* mRNA (Thisted and Gerdes, 1992).

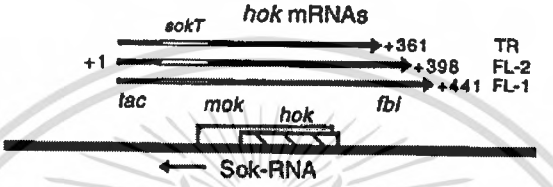


Figure 2.5 Overview of the structural elements of the *hok-sok* TA system from plasmid R1
Source: Franch *et al.* (1997)

Sok-RNA is very unstable, half-life in the order of 30 seconds, but driven by a strong promoter (Thisted *et al.*, 1994b). The *hok* mRNA (Fig.2.6) exists a plasmid carrying cell in two forms, the inert full-length and a shorter active one. In full length *hok* mRNA, the fold back inhibition element (*fbi*) presents at 3' end pairs with 5' end giving an RNA structure inactive both in translation and antisense RNA binding (Thisted *et al.*, 1995). In this form *hok* mRNA is accumulated inside the cell. The full-length stable form is slowly processed at 3' end by polynucleotide phosphorylase and ribonuclease II (Franch and Gerdes, 1996). However, the 3' exoenzyme removes the terminal 39 nt at the 3' end of *hok* mRNA at a low rate. After removal of the *fbi* element, a refolded structure of *hok* mRNA is accessible for antisense RNA binding and for translation. The constitutively expressed 67 nt Sok-RNA recognizes a single stranded stem-loop existing only in the refolded *hok* mRNA. This is because the 3' trimming of full length *hok* mRNA releases the vary 5' end of the mRNA and this release triggers a major refolding of the mRNA 5' end that resulting in the formation of the antisense RNA binding stem-loop structure. The refolded isoform of the mRNA is metabolically very stable and binds Sok-RNA avidly. After initial recognition, Sok-RNA rapidly binds to the translational initiation region (TIR) of *mok* (Gerdes *et al.*, 1992) and more extension duplex formation progresses form the Sok-RNA 5' end is performed by zippering-like mechanism (Fig. 2.7). In addition, formation of this RNA complex leads to immediately RNaseIII, mediated degradation of the mature and translatable of *hok*

mRNA. In plasmid carrying cells, the present of Sok-RNA prevents translation of the truncated mRNA. However, in plasmid free cells, in which Sok-RNA has decayed, the continuous slow processing of the full length mRNA leads to accumulation of the translatable truncated mRNA. Hok protein is small, about 50 amino acids, membrane associated polypeptides. Over expression of Hok leads to decrease of cell membrane potential, arrest of respiration and efflux of small molecules, resulting in cell death.

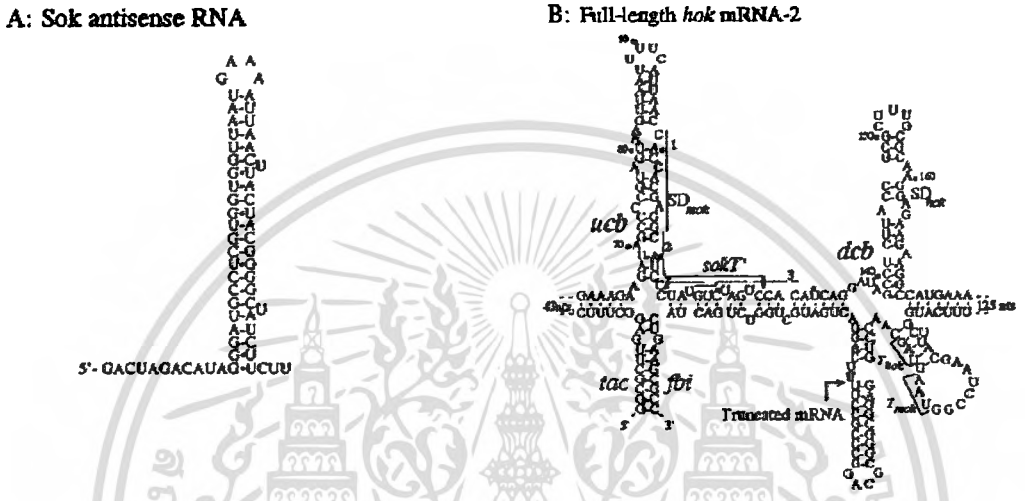


Figure 2.6 Secondary structures of Sok-RNA and *hok* mRNAs.

Source: Franch *et al.* (1997)

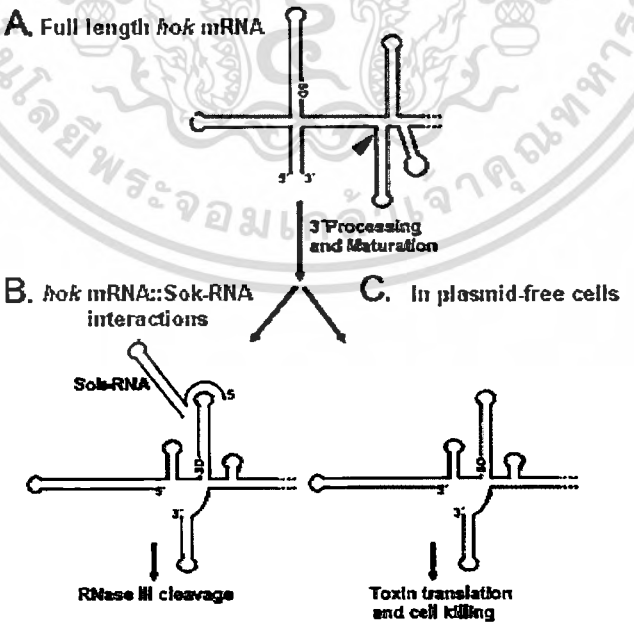


Figure 2.7 Schematic model of the *hok/sok* TA system

Source: Faridani *et al.* (2006)

The *hok-sok* gene homologues have been identified in many replicons as shown in table 2.2. The antisense RNA-regulated stability determinant has been also found in Gram positive bacteria. This determinant, designated *par*, stabilizes the enterococcal plasmid pAD1 and has no homology to the *hok-sok* family (Weaver and Clewell, 1989). The *par* region encodes two small convergently transcribed RNAs with 33 codons for the *fst*, plasmid-stabilizing toxin of *faecalis* sp., peptide inside the longer RNA I. The smaller RNA II shows high degree of complementarity to RNA I. In this complex RNA II inhibis fst translation by preventing ribosome binding to the SD sequence sequestered between complementary direct repeats. Overproduction of RNA I causes host cell death.

Table 2.2 Plasmid and chromosome encoded *hok* homologues

Locus	Replicon	References
hok/sok	R1	Gerdes *et al.*, 1990
flm	F	Loh *et al.*, 1988
*srn*B	F	Ono *et al.*, 1986
pnd	R483	Ono *et al.*, 1987
pnd	R16	Sakikawa *et al.*, 1989
pnd	R64	Furuya and Komano, 1996
*hok*A	*E. coli* C	Pedersen and Gerdes, 1999
*hok*X	*E. coli* B	Pedersen and Gerdes, 1999
*hok*B	*E. coli* K12	Gerdes *et al.*, 1997
*hok*C (*gef*)	*E. coli* ECOR24	Poulsen *et al.*, 1989
*hok*D (*rel*F)	*E. coli* K12	Bech *et al.*, 1985; Gerdes *et al.*, 1986
*hok*E	*E. coli* K12	Gerdes *et al.*, 1997

Source: Zielenkiewicz and Ceglowski (2001)

2.2.2 Protein-regulated cell killing

Protein-regulated cell killing is another type of toxin, encoded by the prokaryotic TA loci, also confers programmed cell death. This system consists of two genes, one encodes a stable toxin and the other encodes an antitoxin protein (Zhao and Zhang, 2008). The toxicity of the lethal toxins is counteracted by a protein antitoxin that combines with and neutralizes the toxin in plasmid containing cells. However, antitoxin is more susceptible to degradation by

cellular protease via *de novo* synthesis in plasmid free cell. Toxin becomes available to induce cell death (Gerdes, 2000). A schematic model of programmed cell death based on toxin and antitoxin system is shown in Fig. 2.8.

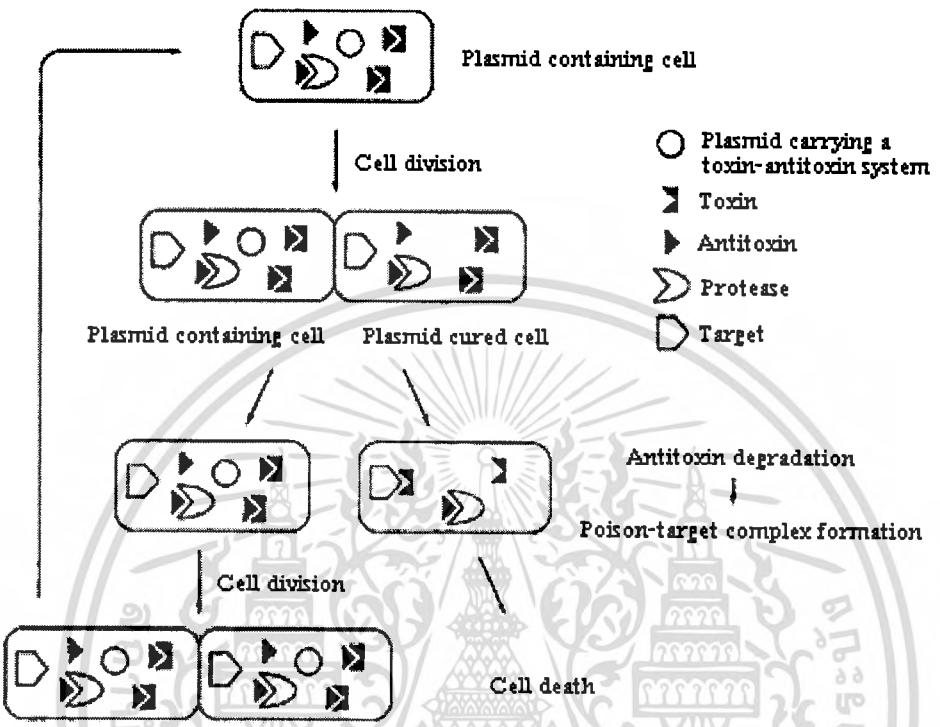


Figure 2.8 A model for the activation of a protein-based toxin-antitoxin system
Source: Couturier *et al.* (1998)

TA modules were first identified on plasmid. During the years, seven plasmids encoded TA families have been described (Table 2.4) (Jensen and Gerdes, 1995). The *ccd* locus of F plasmid was the first to be identified and others were listed in the order of discovery. TA modules have a characteristic gene organization where in most case, the antitoxin component precedes the toxin gene and with the two genes overlapping in their reading frames. One exception to this is the *hig* locus of Rts1 in which the upstream cistron codes for the toxin. A second peculiarity of the *hig* locus is that the toxin is smaller than the antitoxin, whereas the reverse is the case for all other systems known. The distances between the toxin and antitoxin genes vary from locus to locus. In the regular TA operon, a 4 bp gene overlap, in which the upstream antitoxin TGA stop-codon overlaps with ATG start-codon of toxin gene in a ATGA sequence (Pandey and Gerdes, 2005). The genetic organization of known plasmid-encoded TA loci is shown in Fig. 2.9. Antitoxins autoregulate transcription of the TA operon by binding to

operator sites upstream of or overlapping with the operon promotors. In many case, the toxins act as co-repressors of transcription, indicating that a TA complex is bound to the operator sites. Recently, several chromosomal genes similar to plasmid-borne addiction modules have been identified (Brown and Shaw, 2003). The most studied gene among these, *mazEF* was found to located in many bacterial chromosomes and mainly studied in *E. coli*. Another TA module that has been studied extensively is *relBE*.

Table 2.3 Plasmid encoded TA loci

Locus	Organisms	Protease	References
ccd of F	*E. coli*	Lon	Ogura and Hiraga (1983)
parD/pem of R1/R100	*E. coli*	Lon	Bravo *et al.* (1987)
vapBC	*S. Dublin*	unknown	Pullinger *et al.* (1992)
parDE of RK2	Broad-host-range plasmid	unknown	Roberts *et al.* (1992)
phd/doc of P1	*E. coli*	ClpXP	Lehnherr *et al.* (1993)
higBA of Rts1	Broad-host-range plasmid	unknown	Tian *et al.* (1996)
relBE of P307	*E. coli*	Lon	Gronlund *et al.* (1999)

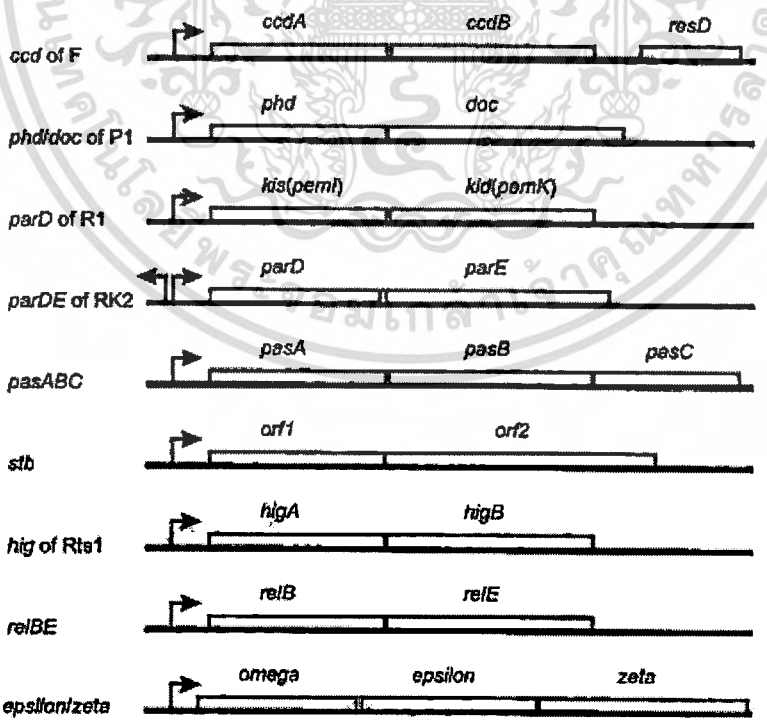


Figure 2.9 Genetic organization of plasmid-encoded TA loci

Source: Gerdes (2000)

2.3 Chromosome-encoded toxin-antitoxin loci

2.3.1 *mazEF* locus

The identical *parD* and *pem* loci of plasmids R1 and R100, respectively, were first identified almost 20 years ago (Bravo *et al.*, 1987). These operons encode identical toxins called Kid and PemK, and their inhibitors, Kis and PemI. Two chromosomal homologues of *pem*, called *chpAK* and *chpBK*, were subsequently identified in *E. coli* (Masuda *et al.*, 1993). The *chpAIK* operon is more commonly referred to as *mazEF* and lies downstream of the stringent factor encoding gene, *relA* (Metzger *et al.*, 1988). The genetic composition of the *mazEF* is shown in Fig. 2.10. *mazF* encodes a stable toxin, MazF, while *mazE* encodes a labile antitoxin, MazE, degraded *in vivo* by the ATP-dependent ClpPA serine protease (Aizenman *et al.*, 1996). The third gene, *mazG*, encoded MazG is a pyrophosphate hydrolase of nucleotides, is located in the *mazEF* operon downstream from *mazF*. Deleting *mazG* decreases cell survival during nutritional stress (Zhang and Inouye, 2002).

Figure 2.10 Genetic composition of the *mazEF*
Source: Pandey and Gerdes (2008)

The structures of many members of the RNA interferase family were solved prior to understanding their function. The crystal structure of *E. coli* MazF was solved in complex with its antitoxin, MazE, shown as Fig. 2.11 (Kamada *et al.*, 2003). Each molecule of a central dimer of MazE extends a long negatively charged random coiled arm around a dimer of MazF, in a hexameric MazF2-MazE2-MazF2 configuration. A model has recently been proposed by Li and colleagues (Li *et al.*, 2006) which the negatively charged C-terminal arm of MazE mimics the similarly charged sugarphosphate backbone of RNA, thus occupying the RNA binding site of the toxin and inhibiting its activity. In this model, however, the antitoxin arm occludes only one of the RNA binding sites of each MazF dimer. The amino acids interacting with a non-cleavable DNA analogue of the RNA substrate have been identified by NMR chemical shift perturbation experiments. These map to two sites, positively charged residues in a flexible loop between strands S1 and S2 of one subunit and a hydrophobic site composed of residues in the loop between strands S3 and S4 of one subunit and helix H1 of the other. A histidine residue (H28) in

the S1-S2 loop is thought to be involved in catalysis. The cleavage mechanism involves an attack by the 2' hydroxyl moiety on the adjacent scissile phosphodiester bond, with the formation of a 2'-3' cyclic phosphate intermediate and release of a downstream cleavage product with a 5' hydroxyl group. The 2'-3' cyclic intermediate can ultimately be resolved into 3' phosphate and 2' hydroxyl groups (Zhang *et al.*, 2005).

Although environmental conditions affecting the expression of the *mazEF* operon were studied for many years in great detail, it only recently became clear that these toxins were endoribonucleases (Christensen and Gerdes 2003). MazF inhibits protein synthesis through its endoribonucleolytic effect on mRNA. MazF endoribonuclease cleaves primarily at single stranded mRNAs at ACA triplets sequences of transfer-messenger-RNA (tmRNA), the tRNA-mRNA hybrids that bind to the A site of ribosomes containing a truncated mRNA, tagging the corresponding nascent polypeptide chains with a degradation signal, while allowing translation to terminate normally (Karzai *et al.*, 2000). Further cleavage of mRNAs and tmRNA by MazF would be prevented by MazE, and the previously truncated mRNAs could be released form the ribosomes through the action of de novo synthesized uncleaved tmRNA. However, MazE cannot reverse the downstream cascade already initiated by MazF (Engelberg *et al.*, 2005).

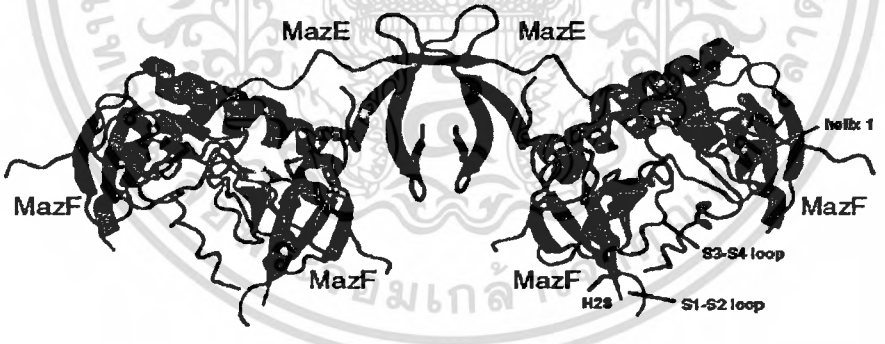


Figure 2.11 Structures of RNA cleaving toxins and their inhibitors of *E. coli* MazEF complex (PDB code 1UB4). Toxin subunits are in different shades of green, antitoxin subunits are in red or orange.

Source: Kamada *et al.* (2003).

mazEF located in many of bacterial chromosomes and mainly studied in *E.coli*. However, *mazEF*-like modules occur in the chromosomes of many other bacteria including pathogens (Gerdes *et al.*, 2005). *M. tuberculosis* is a devastating pathogen in which there may be

functional MazF homologs (Zhu *et al.*, 2006). The chromosome of *M. tuberculosis* bears at least seven genes encoding MazF-like products cause cell death when ectopically expressed in *E.coli.*

2.3.2 *relBE* locus

The *relBE* locus is also found to be widely spread among eubacteria, including gram positive and gram negative species, and archea (Gerdes, 2000). The *relBE* gene was first identified from *E.coli* and encodes three genes, *relB*, *relE* and *relF*, (Bech *et al.*, 1985). Genetic structure of *E.coli relBE* operon is shown as Fig. 2.12.

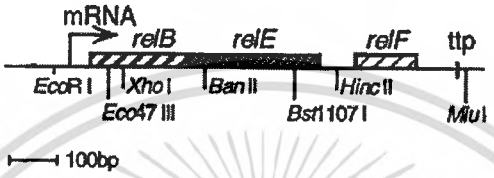


Figure 2.12 Genetic structure of the *relBE* operon of the *E. coli* map
Source: Gotfredsen and Gerdes (1998)

The antitoxin gene, *relB*, is located upstream of the toxin gene, *relE*, and both are translated from one mRNA transcript (Diderichsen *et al.*, 1977). RelB, encoded from *relB* gene, is a 9.1 kDa acidic protein which form a strong complex and acts to neutralize with RelE, encoded from *relE* gene, by direct protein-protein interaction (Sevin and Barloy-Hubler, 2007). Furthermore, RelB autoregulates transcription of *relBE*, and the RelB-RelE complex yields even better repression than RelB alone. Thus, RelE is a co-repression of *relBE* transcription. During balanced growth RelB is expressed in excess over RelE (Gotfredsen and Gerdes, 1998) but if *de novo* synthesis of RelB, which is unstable due to degradation by Lon proteases, becomes too small, its concentration will dwindle, free and active RelE toxins will appear in the cytoplasm (Christensen *et al.*, 2001). RelE is an 11.2 kDa basic protein and is cytotoxic or cytostatic when overexpressed in bacteria. Overexpression of *relE* restored protein synthesis and colony formation by cleaving mRNA codons in the ribosomal A site in a sequence specific way with preference for the second and third base of stop codon. Among stop codons UAG is cleaved with fast, UAA intermediate and UGA slow rate, while UCG and CAG are cleaved most rapidly among sense codons (Pederson *et al.*, 2003). The third gene of *relBE* operon, *relF*, encodes a cytotoxin that belongs to the Hok family of protein and leads to rapid cessation of cell growth, arrest of respiration and collapse of the cell membrane potential (Gerdes *et al.*, 1986). Therefore, the *relF* gene was renamed *hokD* (Gerdes *et al.*, 1997). The physiological significance of the RelF protein

is not known, but the *relF* cistron is not translated during steady state cell growth and does not contribute to plasmid stabilization (Gotfredsen and Gerdes, 1998).

Recently, the crystal structure of RelB-RelE, antitoxin-toxin complex, from hyperthermophilic archaeon *Pyrococcus horikoshii* were also solved by Takagi and colleagues (Takagi *et al.*, 2005). $RelB_2$-$RelE_2$ is a tetrameric complex which the protein folds are clearly distinct from of the RNA interferases and their antitoxin shown as Fig. 2.13. Competition between translation release factor RF1 and RelE for ribosome binding suggested that RelE enters the A-site of the ribosome to cleave RNA (Pedersen *et al.*, 2003). Indeed Takagi and colleagues pointed out that the structure of RelE has a similar overall shape and dimension to the decoding domain of elongation factor G (EFG), which also enters the A-site (Takagi *et al.*, 2005). They propose that the wrapping of the RelB antitoxin around RelE increases its bulk and sterically prevents it from entering the A-site, rather than competing with RNA binding. Indeed, an alanine scanning mutagenesis of conserved amino acids pointed to a series of arginine residues on one face of RelE as a possible functional site, relatively unhindered by the interaction with the antitoxin. However, the sequence similarity between the archaeal *P. horikoshii* RelB and bacterial *E. coli* RelB is relatively low, with 24% identity and 48% similarity. High level of homology only resides within the C-terminal antitoxin. The sequence variation in the N-terminus indicates that *E.coli* RelB may employ a different method for the transcriptional regulation (Li *et al.*, 2008).

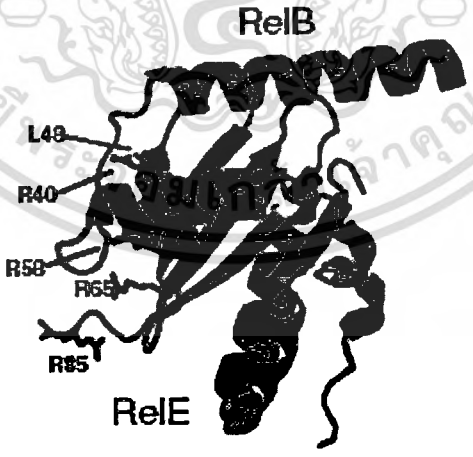


Figure 2.13 Structures of RNA cleaving toxins and their inhibitors of RelBE complex (PDB Code 1MW1) showing amino acids involved in catalysis. Toxin subunits are in different shades of green, antitoxin subunits are in red or orange.

Source: Takagi *et al.* (2005)

2.4 Stress response of TA loci

The mechanism of stress response of TA loci in many bacteria has remained unclear, but experimental observations suggested that it targets a factor involved in protein synthesis (Galvani *et al.*, 2001). In *E.coli*, experimental evidence indicates that *mazEF* and *relBE* are stress response elements that help cells survive unfavorable growth condition such as amino acid or carbon source starvation (Pandey and Gerdes, 2008). Carbon source starvation leads to alter expression rates of a large number of genes (Nyström, 1994), and amino acid starvation leads to arrest of synthesis of stable RNA, rRNA and tRNA, by increasing rate of guanosine. Guanosine-5'-triphosphate-3-diphosphate (pppGpp) and guanosine-3',5'-bis-pyrophosphate (ppGpp) are synthesized by ribosome associated enzyme encoded by the *relA* gene, ppGpp synthetase I (Galvani *et al.*, 2001). The accumulation of (p)ppGpp inhibits exopolyphosphatase (PPX) while increases polyphosphate kinase (PPK) that generates inorganic phosphate (PolyP) (Christensen and Gerdes, 2004). The increasing of PolyP activates Lon protease towards a subset of idling ribosomal proteins, ultimately leading to the generation of free amino acid, which can be used for *de novo* protein synthesis (Kuroda *et al.*, 2001). Under condition of amino acid starvation, *de novo* synthesis of MazE and RelB are blocked and degraded by Lon protease. This results in both transcriptional derepression of the *mazEF* and *relBE* operons and activation of MazF and RelE ribonuclease functions. MazF can cleave mRNAs between ribosomes or RNAs not yet engaged in translation while RelE promotes cleavage of mRNAs at sites of ribosome stalling by binding to the ribosome A-site. The increase in *mazEF* and *relBE* expression was shown to be dependent on Lon protease and independent of *relA*, suggesting that *mazEF* and *relBE* induction mechanism is also via a release of autorepression. Upon restoration of amino acid levels, stalled ribosomes are rescued by tmRNA and renewed synthesis of MazE and RelB blocks MazF and RelE activity once again, allowing resumption of cell growth adjusted to the new amino acid levels. Effect of amino acid starvation on MazEF and RelBE activity and expression is shown as Fig. 2.14.

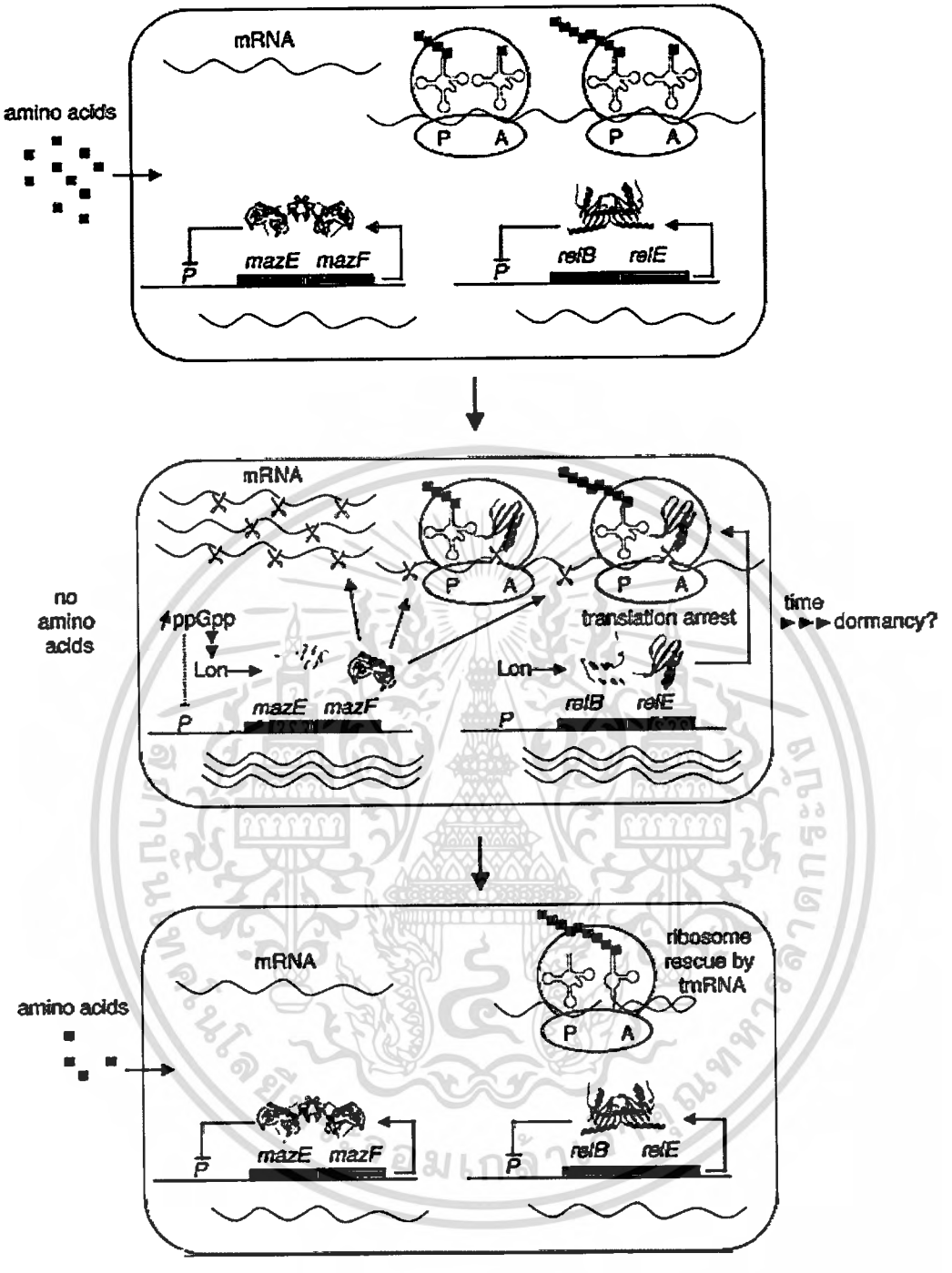


Figure 2.14 Effect of amino acid starvation on MazEF and RelBE activity and expression

Source: Condon (2006)

E. coli tmRNA has two known functions. It releases stalled ribosomes from damaged mRNAs and tags the nascent polypeptides from such ribosomes for proteolysis (Muto *et al.*, 1998). Thus, tmRNA acts as both a tRNA and mRNA. tmRNA also encodes a short tag sequence ANDENYALAA (Tu *et al.*, 1995). It is believed that tmRNA enters the empty A-site of stalled

ribosomes and adds its charged alanine to the nascent polypeptide. Translation then shifts from the original mRNA to the ANDENYALAA reading frame within tmRNA, a process called *trans*-translation. As a result, the stalled ribosome is rescued from the damaged mRNA, and the tmRNA-encoded peptide tag is added to the C-terminus of the nascent polypeptide. The tmRNA tag is recognized by multiple proteases, resulting in rapid degradation of the tagged proteins (Keiler *et al.*, 1996). Activation of RelE should result in stalled ribosomes on damaged mRNAs. Therefore, tmRNA might be involved in recovery of RelE-inhibited cells. Overexpression of tmRNA counteracted RelE toxicity, and tmRNA stimulated restart of translation in RelE-inhibited cells. Hence, tmRNA rescues ribosomes stalled on mRNAs cleaved by RelE. The stress response scheme of *relBE* is shown as figure 2.15.

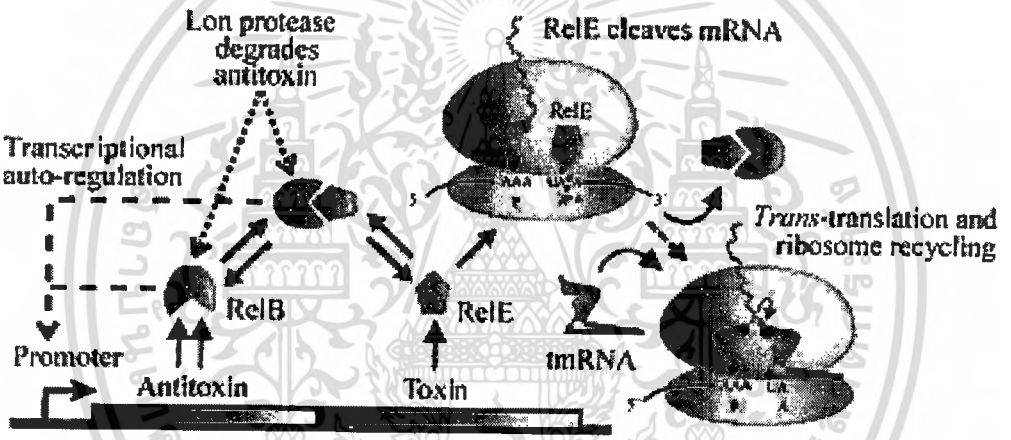


Figure 2.15 Overview of the *trans*-translation by tmRNA in *relBE* locus

Source: Christensen and Gerdes (2004)

The other stress conditions such as thymine starvation, mitomycin C, nalidixic acid, UV radition and oxidative stress (H_2O_2) also trigger cell death. Sat and colleagues have shown that Δ*mazEF* cells are significantly more resistant to a wide variety of stress condition than wild type *E. coli* cells. Theses include relatively short exposure to antibiotics that specifically interfere with transcription or translation, such as rifampicin, spectinomycin and chloramphenicol (Sat *et al.*, 2003), conditions causing DNA damage, such as thymine starvation (Sat *et al.*, 2001), mitomycin C, nalidixic- acid and UV irradiation, and other conditions including high temperature and oxidative stress (Hazan and Engelberg, 2004). Thymine starvation was shown to have a negative effect on *mazEF* expression, similar to that observed by overproduction of RelA (Sat *et al.*, 2003). Addition of chloramphenicol to cultures caused a similar increase in *relBE* expression, suggesting that the signal for derepression is translation arrest. Glucose starvation also

resulted in increased *relBE* expression, albeit to a lesser extent, whereas heat-shock at 42°C did not affect expression. Interestingly, the stress conditions listed above generally affected log phase cells growing on rich medium much more than stationary phase cells or cells growing on minimal medium (Sat *et al.*, 2001), suggesting that it is easier to do irreparable damage to cells when they are growing flat out and least expect it. Stress conditions also affected wild-type cells much more than *relA* mutant cells, suggesting that ppGpp plays a role in this phenomenon. Overexpression of *mazF* and *relE* confer inhibition of cell growth, reduction in the number of colony-forming cells and severe inhibition of translation (Gotfredsen and Gerdes, 1998). However, it might be advantageous for a whole cell population. For example, *mazEF*-mediated death can act as a defense mechanism that prevents the spread of phages (Diderichsen *et al.*, 1977).

TA loci are also present on bacterial chromosome often in multiple copies such as the chromosome of *E. coli* K-12 encodes three *relBE* homologous loci (Anantharaman and Aravind, 2003) and two *mazEF* homologous loci. The model organism *E.coli* K-12 is shown as figure 2.16(A). Chromosomal TA loci have only been studied in a few organisms other than *E. coli*. An exhaustive search for TA loci in the genomes of 126 fully sequenced prokaryotic organisms revealed 671 candidate gene pairs belonging to seven TA families (Pandey and Gerdes, 2005). Of these, 10% are RNA interferases and 23% belong to the RelBE family, with 42% VapC homologues having the greatest representation. While MazF homologues are restricted to bacteria, RelE homologues are widely distributed in both bacteria and archaea. The number of TA loci in a single genome can range from none, in the obligate intracellular parasites, to dozens. *Nitrosomonas europa*, for example, has four MazEF and ten RelBE homologues out of a total of 43 TA *loci* (Diderichsen *et al.*, 1977). It has been proposed that the uneven distribution of TA loci reflects the environment obligate intracellular parasites live in a much more constant environment than free living organisms and therefore do not need such stress management mechanisms. Seven MazF homologues and three RelBE homologues have been identified in *M. tuberculosis* H37Rv. Chromosomal maps of TA loci *M. tuberculosis* H37Rv is shown as figure 2.16(B). Four of MazF homologues have been shown to cause varying degree of toxicity in *E.coli* (Zhu *et al.*, 2006). The cleavage specificities of MazF-mt1 and mt6 are UAC and U-rich sequences respectively. The expression of two of these genes encoding MazF homologue, MazF-mt4 and mt6, is directly or indirectly downregulated by ppGpp synthesis (Betts *et al.*, 2002). RelE proteins from Gram-positive Bacteria and Archaea cleaved tmRNA with a pattern similar to that of *E. coli* RelE,

suggesting that the function and target of RelE may be conserved across the prokaryotic domains. However, the stress response function of RelE in *M. tuberculosis* is still not clearly known.

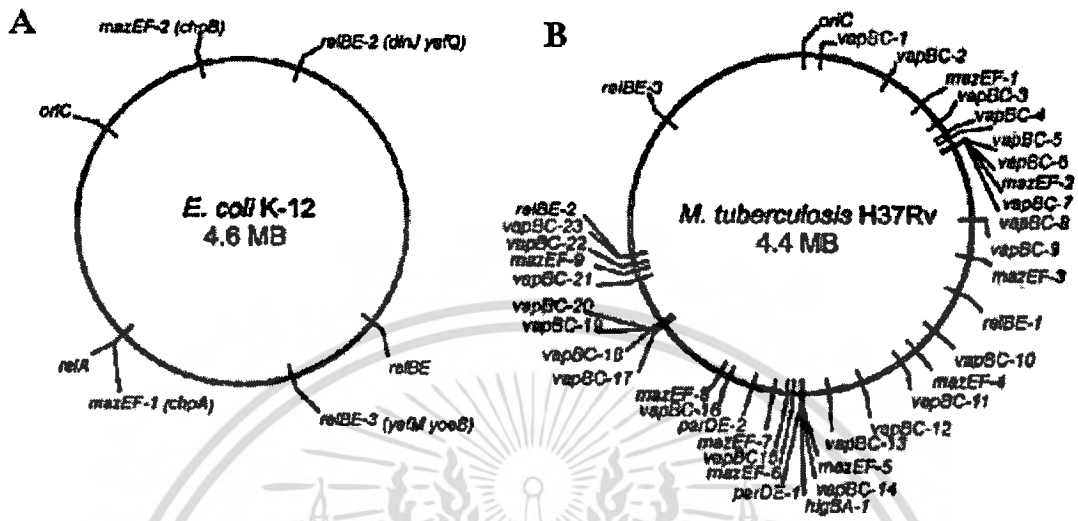


Figure 2.16 Chromosomal maps of TA loci in *E.coli* and *M. tuberculosis*

Source: Pandey and Gerdes (2005)

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial strains

3.1.1 *Mycobacterium tuberculosis* H37Rv ATCC 25618 was obtained from Dr. Therdsak Prammananan, National Center for Genetic Engineering and Biotechnology, Thailand

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

3.1.3 *Escherichia coli* strain DH5α (*supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) (Invitrogen, Carlsbad, USA)

3.2 Plasmids

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

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

3.3 Chemical reagents

3.3.1 Culture medium

3.3.1.1 Luria-Bertani (LB) medium (Appendix B) (BBL, Becton-Dickinson, USA)

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

3.3.1.3 SOB medium (Appendix B) (Invitrogen, Carlsbad, USA)

3.3.2 Antibiotics

3.3.2.1 Kanamycin (Amersham Bioscience, Piscataway, USA)

3.3.2.2 Ampicillin (USB, Cleveland, USA)

3.3.3 Enzymes

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

3.3.3.2 Lysozyme (Sigma, St. Louis, USA)

3.3.3.3 *Bam*HI (Promega, Madison, USA)

3.3.3.4 *Cla*I (Promega, Madison, USA)

3.3.3.5 *Eco*RI (Promega, Madison, USA)

3.3.3.6 *Xba*I (Promega, Madison, USA)

3.3.3.7 *Spe*I (Promega, Madison, USA)

3.3.3.8 T4 DNA ligase (Amersham Bioscience, Piscataway, USA)

3.3.4 DNA markers

3.3.4.1 λDNA/*Hind* III fragments (Invitrogen, Carlsbad, USA)

3.3.4.2 100 bp Ladder DNA (Invitrogen, Carlsbad, USA)

3.3.5 Chemicals

3.3.5.1 Agarose (Sigma, St. Louis, USA)

3.3.5.2 Bacteriological agar (Scharlau, Barcelona, Spain)

3.3.5.3 Boric acid (Merck, Darmstadt, Germany)

3.3.5.4 Calcium chloride (Scharlau, Barcelona, Spain)

3.3.5.5 dNTPs (deoxynucleotide triphosphates) (Promega, Madison, USA)

3.3.5.6 EDTA (Ethylenediaminetetraacetic acid) (Bio Basic, Ontario, Canada)

3.3.5.7 Ethanol (Fisher, Springfield, USA)

3.3.5.8 Gel star (Cambrix Bio Science Rockland, Rockland, USA)

3.3.5.9 Glacial acetic acid (Labscan, Dublin, Ireland)

3.3.5.10 Glycerol (Fluka, Buchs, Schweizerland)

3.3.5.11 Hydrochloric acid (Labscan, Dublin, Ireland)

3.3.5.12 Hydrogen peroxide (Sigma, St. Louis, USA)

3.3.5.13 IPTG (Isopropyl-β-D-thiogalactopyranoside) (Sigma, St. Louis, USA)

3.3.5.14 Manganese chloride (Scharlau, Barcelona, Spain)

3.3.5.15 MOPs (3-(N-morpholino)propanesulfonic acid) (Sigma, St. Louis, USA)

3.3.5.16 Potassium chloride (Scharlau, Barcelona, Spain)

3.3.5.17 Potassium acetate (Fisher, Springfield, USA)

3.3.5.18 Sodium acetate (Scharlau, Barcelona, Spain)

3.3.5.19 Sodium chloride (Labscan, Dublin, Ireland)

3.3.5.20 Sodium dodecyl sulfate (Promega, Madison,USA)

3.3.5.21 Sodium hydroxide (Labscan, Dublin, Ireland)

3.3.5.22 Sodium nitrate (Sigma, St. Louis, USA)

3.3.5.23 Tris (USB, Cleveland, USA)

3.3.5.24 Tween 80 (Fluka, Buchs, Schweizerland)

3.3.5.25 X-gal (5-Bromo-4-chloro-indoLyl-β-D-galactopyranoside) (Sigma, St. Louis, USA)

3.3.6 Kits

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

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

3.4 Instruments

3.4.1 Autoclave (autoclave-325, Tomy, Japan)

3.4.2 Centrifuge (Z383K, Hermle-Labortech Inc., Germany)

3.4.3 DNA thermal cycler (Perkin Elmer, Scientific Support Inc., USA)

3.4.4 Documentation gel analysis (Bts-20.M, Syngene, Germany)

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

3.4.6 Glass wares

3.4.7 Incubator (Binder control, Scientific Promotion, Japan)

3.4.8 Incubator shaker (Innova, New Brunswick Scientific, USA)

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

3.4.10 Microcentrifuge (spectrafuge, Labnet Inc., Germany)

3.4.11 pH meter (215, Denver Instrument, USA)

3.4.12 Power supply (EPS 301, Amersham Pharmacia Biotech, Sweden)

3.4.13 Thermoblock (TDB-120 Thermostat, Biosan, Germany)

3.4.14 Vortex (Genie 2, Scientific Industries, USA)

3.5 Methods

Part I : Construction of the recombinant plasmid pMV261 containing *relE* gene

The recombinant plasmid pMV261 containing *relE* gene of *M. tuberculosis* H37Rv was constructed by two cloning steps. First, *M. tuberculosis relE* gene was amplified by PCR using

crude DNA as a template. PCR product was purified by QIAgel extraction kit and ligated to the TA-cloning vector pDrive. The recombinant plasmid was transformed into the competent cell *E. coli* DH5α and transformants were selected on 50 μg/ml of kanamycin, 40 μg/ml of X-gal and 0.1 mM of IPTG containing LB agar by blue-white screening. Plasmid DNAs were isolated from the white colonies and sequenced. Second, *relE* gene of *M. tuberculosis* in the recombinant plasmid pDrive-*relE* was subcloned into the *E. coli* / mycobacterium shutter vector pMV261. The recombinant plasmid pMV261-*relE* was transformed into the competent cell *M. smegmatis* mc^2155 and transformants were selected on 50 μg/ml of kanamycin containing LB agar.

3.5.1 Growth conditions

E. coli was grown in LB (Appendix B) agar at 37°C for overnight or LB broth with the shaking speed of 250 rpm at 37 °C. *M. smegmatis* was grown in LB broth supplemented with 0.05% (w/v) Tween 80 at 37 °C with the shaking speed of 200 rpm for 2-3 days. *M. tuberculosis* was grown in the Middlebrook 7H10 (Appendix B) agar at 37°C for 3-4 weeks. For selection of recombinant clones, the medium was supplemented with kanamycin at the final concentration of 50 μg/ml.

3.5.2 Crude DNA isolation of *M. tuberculosis*

One loopful of *M. tuberculosis* H37Rv colonies was scraped from the agar plate and resuspended in 200 μl of TE buffer. Cell suspension was boiled for 20 min before centrifugation at 13,000 rpm for 3 min. Crude DNA was removed and stored at -20 °C.

3.5.3 Primer design for amplification *relE* gene of *M. tuberculosis* by polymerase chain reaction (PCR)

The sequence of *M. tuberculosis relE* was derived from the Genbank database (Cole *et al.*, 1998). The PCR primers were designed to contain the restriction enzyme recognition sites at 5′ end to facilitate a directional cloning. The sequence of the forward primer, FB2866, was 5′-GC**GGATCC**ATGCCTTACACCATGCG-3′ (Figure 3.1). The 5′ end of forward primer FB2866 contained the recognition site *Bam*HI (GGATCC). The sequence of the reverse primer, RC2866, was 5′-CG**ATCGAT**GGTGAGTTGCTATCGGCG-3′ (Fig. 3.1) and the end of this primer contained the recognition site of *Cla*I (ATCGAT).

FB 2866 primer : 5′-GC**GGATCC**ATGCCTTACACCATGCG-3′

Start

GC**TGAGCG**ATG CCTTACACC**A TG**CGGTTCAC CACAACCGCG CGTCGAGACC

TCCACAAGCT GCCACCGCGC ATCCTCGCGG CAGTGGTCGA ATTCGCGTTC

GGCGATCTGT CGCGCGAGCC CCTGCGGGTG GGCAAGCCCC TTCGGCGCGA

GTTGGCCGGC ACGTTCAGCG CGCGTCGCGG AACGTACCGC CTGCTGTACC

Stop

GGATTGACGA CGAGCACACA ACGGTAG**TGA** TCCTGCGCGT CGATCACCGC

GCGGACATCT AC**C**GCCGATA GCAACTCACC **GACGGCG**

RC 2866 primer : 5′-CG**ATCGATG**GTGAGTTGCTATCGGCG-3′

Figure 3.1 Sequences of *M. tuberculosis relE* gene and location of primer FB2866 and RC2866

3.5.4 DNA amplification of *M. tuberculosis relE* by PCR

M. tuberculosis relE was amplified by polymerase chain reaction (PCR) with primer FB2866 and RC2866 using crude DNA of *M. tuberculosis* as a template. The 50 µl of PCR reaction was performed in a 500 µl PCR tube. The composition of PCR reaction was shown in table 3.1. The reaction parameters consisted of an initial denaturation step at 94 °C for 5 min followed by 40 amplification cycles of a denaturation step at 94 °C for 1 min, annealing step at 50 °C for 1 min, and an extension step at 72 °C for 2 min. The final cycle was followed by an additional extension step at 72 °C for 10 min.

Table 3.1 PCR reaction of *M. tuberculosis relE*

Components	Volume (µl)
10x PCR buffer	5
10 mM dNTPs	1
5 µM FB2866 forward primer	2.5
5 µM RC2866 reverse primer	2.5
25 mM $MgCl_2$	3
Taq DNA polymerase (5 units/µl)	0.5
Crude DNA of *M. tuberculosis*	5
Deionized water	30.5
Total volume	50

3.5.5 Analysis of PCR product by agarose gel electrophoresis

A 206-bp *relE* PCR product was detected by running on 1% agarose gel. Of 20 ml, TBE buffer, 0.2 g of agarose was added and heated. Gel star® in dilution of 1:100 was added to the worm agarose solution. Five microlitres of PCR products and 500 ng of λDNA/*Hind* III fragments were loaded into the wells. The electric current of 8 V/cm was applied to the gel. After electrophoresis, the gel is illuminated under an ultraviolet light.

3.5.6 DNA fragments purification

DNA fragments were purified by using QIAquick gel extraction kit (Qiagen, Germany). According to the manufacturer's instructions, DNA fragments were excised from agarose gel and transferred to microcentrifuge tubes. QG buffer with 3 vol of gel weight was added and incubated at 50 ^{0}C for 10 min or until gel was dissolved. After the gel slice has dissolved completely, the gel solution was transferred into QIAquick column. The column was centrifuged at 13,000 rpm for 1 min and flow through was discarded. To wash the column, 750 µl of buffer PE was added and centrifuged at 13,000 rpm for 1 min. The flow through was discarded and the column was centrifuged for an additional 1 min at 13,000 rpm. QIAquick column was placed into a microcentrifuge tube. To elute DNA, 30 µl of buffer EB was added to the center of the QIAquick membrane and the column was centrifuged for 1 min. The purified DNA was analyzed by gel electrohoresis (section 3.5.5).

3.5.7 Ligation of *relE* PCR product to TA-cloning vector

The purified PCR product of *M. tuberculosis relE* gene as described in 3.5.6 was ligated to the plasmid pDrive (Qiagen, Germany) by setting up a ligation reaction as shown in table 3.2. The ligation reaction was incubated at 20 °C for 4 hours and transformed into the competent cell *E. coli* strain DH5α.

Table 3.2 Ligation reaction of *relE* PCR product and plasmid pDrive

Component	Volume (µl)
Plasmid pDrive (50 ng/ µl)	1
Purified PCR product of *relE* (16 ng/ µl)	4
2 x ligation buffer containing T4 Ligase (0.3 units/ µl)	5
Total volume	10

3.5.8 Competent cells preparation

A single colony of *E. coli* DH5α was picked up from LB agar plate and transferred into 5 ml of LB broth. The culture was incubated at 37 °C with shaking speed of 250 rpm for overnight. Three millilitres of overnight culture was diluted into 100 ml of SOB medium (Appendix B) in a 250 ml flask and incubated at 37 °C with shaking speed of 250 rpm for 2 to 3 hours or until reach OD_{600} of 0.6-0.8. Subsequently, cells were stored on ice for 15 minutes and harvested by centrifugation at 5,000 rpm for 15 minutes at 4 °C. RF1 solution (10 mM KCl, 50 mM $MnCl_2$, 30 mM KOAc and 10 mM $CaCl_2$) was added into cell pellets for resuspension in one-third volumes of SOB medium. The cell suspension was incubated for 15 minutes on ice and recovered by centrifugation at 5,000 rpm for 15 minutes. RF2 solution (10 mM MOPs, 10 mM KCl and 75 mM $CaCl_2$) was added into cell pellets for resuspension in one-twenty fifth volumes of SOB medium. Cell suspension was transferred to microcentrifuge tubes in small aliquots and stored at -70 °C.

3.5.9 Transformation of competent cell *E. coli* DH5α

The ligation reaction as described in section 3.5.7 was mixed with 100 µl of competent cells *E. coli* DH5α and incubated on ice for 30 minutes. The suspension was subsequently heated-shock at 42 °C for 90 sec following incubated on ice for 2 min. Nine-hundreds microlitres of LB broth was added and the culture was incubated at 37 °C for 1 h. One-hundred microlitres of suspension was spreaded on LB agar containing 50 µg/ml of kanamycin and incubated at 37 °C for overnight.

3.5.10 Plasmid DNA isolation by QIAprep Spin Miniprep Kit

One colony of transformants was inoculated into 5 ml of LB broth supplemented with 50 µg/ml of kanamycin and incubated at 37 °C with shaking at 250 rpm for overnight. Cells were harvested by centrifuged at 13,000 rpm for 1 min and resuspended in 250 µl of buffer P1. Two-hundreds and fifty microlitres of buffer P2 was added and gently mixed by inversion. After that, 350 µl of buffer N3 was added and the suspension was immediately mixed by inversion. Cell debris was pelleted by centrifugation at 13,000 rpm for 10 min and supernatant was transferred to QIAprep spin column. The column was centrifuged at 13,000 rpm for 1 min and flow through was discarded. To wash the column, 750 µl of PE buffer was added and the column was centrifuged at 13,000 rpm for 1 min. The flow through was discarded and the column was recentrifuged again for 1 min. The column was placed in a new microcentrifuge tube and

50 µl of buffer EB was added to center of the column. After that, the tube was centrifuged at 13,000 rpm for 1 min. Plasmid DNA was eluted and analyzed by 0.8% agarose gel electrophoresis.

3.5.11 Recombinant plasmid DNA digestion by restriction enzyme

The recombinant plasmid, pDrive-*relE* was examined by restriction enzyme *Eco*RI digestion. The digestion reaction was described in table 3.3 and incubated at 37 °C for overnight and then analyzed by 1% agarose gel electrophoresis.

Table 3.3 Digestion reaction of pDrive-*relE* by restriction enzyme *Eco*RI

Components	Volume (µl)
pDrive-*relE* (70 ng/µl)	5
*Eco*RI (12 units/µl)	1
10 x *Eco*RI Buffer	1
BSA (0.1 mg/ml)	1
Deionized water	2
Total volume	10

3.5.12 Nucleotide sequencing

The cloned PCR product was sequenced in both strands with the Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) by using an ABI PRISM® 3700 DNA analyzer. Universal primers T7 and SP6 were used for the sequencing reaction.

3.5.13 Subcloning of *relE* to *E. coli* / Mycobacterium shuttle vector pMV261

Plasmid pDrive-*relE* and pMV261 were prepared by QIAprep Spin Miniprep Kit as described in section 3.5.10. They were digested by restriction enzymes *Bam*HI and *Cla*I and digestion reaction was shown in table 3.4. The reaction was incubated at 37 °C for overnight. DNA fragments of *relE* and pMV261 were purified by using QIAquick gel extraction kit as described in 3.5.6 and analyzed by 2% agarose gel electrophoresis.

Table 3.4 Digestion reaction of pDrive-*relE* and pMV261 with restriction enzyme

Components	Volume (µl)	
	pDrive-*relE*	pMV261
pDrive-*relE* (50 ng/µl)	30	-
pMV261 (50 ng/ µl)	-	30
*Cla*I (12 units/µl)	3	3
*Bam*HI (12 units/µl)	3	3
10 x Buffer	6	6
BSA (0.1 mg/ml)	6	6
Deionized water	12	12
Total volume	60	60

3.5.14 Ligation of pMV261 with *M. tuberculosis relE*

The purified DNA fragment of *relE* gene was ligated into *Cla*I-*Bam*HI digested pMV261. The ligation reaction was shown in table 3.5 and incubated at 20 °C for 4 h. After incubation, ligation reaction was transformed into the competent cell *E. coli* DH5α as described in 3.5.9.

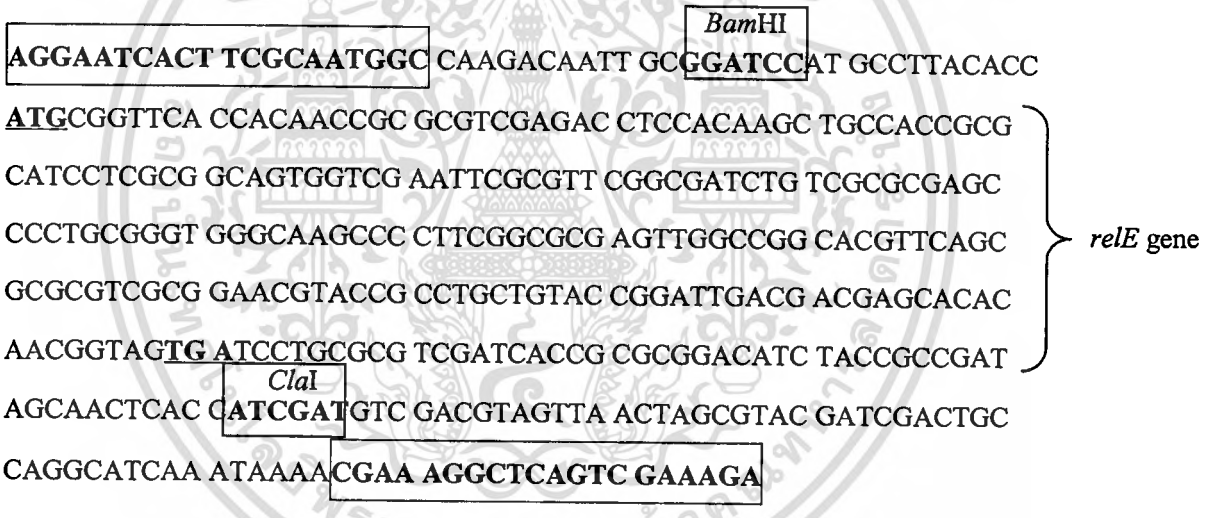
Table 3.5 Ligation reaction of *Cla*I-*Bam*HI digested pMV261 with *M. tuberculosis relE*

Components	Volume (µl)
Purified DNA fragment of *relE* gene (50 ng/µl)	4
*Cla*I-*Bam*HI digested pMV261 (50 ng/µl)	3
T4 ligase (30 units/ µl)	1
10 x ligation buffer	1
30% PEG 8,000	1
Total volume	10

3.5.15 Transformants analysis by PCR

Transformants containing the recombinant plasmid pMV261-*relE* was detected by PCR. PCR primers pMV261-FBam and pMV261-RCla, were designed from nucleotide sequences of pMV261 at upstream and downstream positions of the cloning sites of *relE*. Their sequences and positions were shown in Fig. 3.2. Crude DNA of transformant was isolated according to section 3.5.2 and used as a template in a PCR reaction. The PCR reaction was done as described in table 3.6. The reaction parameters consisted of an initial denaturation step at 94 °C for 5 min followed by 40 amplification cycles of a denaturation step at 94 °C for 1 min, annealing step at 50 °C for 1 min, and an extension step at 72 °C for 2 min. The final cycle was followed by an additional extension step at 72 °C for 5 min.

pMV261-FBam forward primer : 5′- AGGAATCACTTCGCAAT -3′



pMV261-RCla reverse primer : 5′- CGAAAGGCTCAGTCGAAAGA -3′

Figure 3.2 Sequence of *relE* including upstream and downstream regions of recombinant plasmid pMV261-*relE*. Primer locations and sequences are shown in boxes.

Table 3.6 PCR reaction for detection recombinant plasmid pMV261- *relE*

Components	Volume (µl)
10x PCR buffer	5
10 mM dNTPs	1
5 µM pMV261-FBam forward primer	2.5
5 µM pMV261-RCla reverse primer	2.5
25 mM $MgCl_2$	3
Taq DNA polymerase (5 units/µl)	0.5
Crude DNA	5
Deionized water	30.5
Total volume	50

3.5.16 Plasmid DNA digestion with restriction enzymes

After detection by PCR, the recombinant plasmids of selected transformants were isolated by QIAprep Spin Miniprep Kit as described in section 3.5.10. They were rechecked by a restriction enzyme digestion. Theoretically, the recombinant plasmid pMV261-*relE* was digested with restriction enzymes *Eco*RI and *Xba*I resulting in two DNA fragments of 600 and 3,600 bp. The digestion reaction was described in table 3.7. and incubated at 37 °C for overnight.

Table 3.7 Digestion reaction of pMV261-*relE* with restriction enzyme *Eco*RI and *Xba*I

Components	Volume (µl)
pMV261-*relE* (50 ng/µl)	5
*Eco*RI (12 units/µl)	0.5
*Xba*I (12 units/µl)	0.5
10 x Multicore Buffer	1
BSA (0.1 mg/ml)	1
Deionized water	2
Total volume	10

3.5.17 Transformants detection by nucleotide sequencing

The recombinant plasmid was sequenced in both strands at the *relE* gene by using ABI PRISM^{R} 3700 DNA analyzer (Perkin Elmer, USA) with either pMV261-FBam or pMV261-RCla primers for pMV261-*relE*.

3.5.18 Electrotransformation into *M. smegmatis* mc^{2}155

Competent cell of *M. smegmatis* mc^{2}155 was prepared by inoculating one colony of *M. smegmatis* mc^{2}155 in 20 ml of LB medium containing 0.05% Tween 80 and incubated at 37 °C with a shaking speed at 200 rpm for 3-5 days. The large-scale culture (200ml) is prepared by inoculating 10 ml of pre-culture into 200 ml of LB broth and incubated at 37 °C with shaking for 17 h or until an OD_{600} reached 0.5 to 1.0. Cells were incubated on ice for 1.5 hours before harvesting by centrifugation at 5,000 rpm 4 °C for 10 min. The cells were washed three times in ice-cold 10% glycerol. Finally, cells were resuspended in 1:100 original culture volume of ice-cold 10% glycerol. Approximate 1 μg of recombinant plasmid DNA was mixed with 100 μl of competent cells. The cell suspension was kept on ice for 5 min and transferred to a 0.2 cm electrode-gap electroporation cuvette. The cuvette was placed on electroporation chamber and subjected to one single pulse of 2.5 kV, 25 μF with the pulse-controller resistance setting at 1,000 ohms. After electroporation, cuvette was standed on ice for 10 min and added with 1 ml of LB broth. The suspension was then transferred to a sterile 15 ml tube and incubated at 37 °C for 2 hours. A total of 200 μl of cell suspension was spreaded on LB plate containing 50 μg/ml kanamycin and incubated at 37 °C for 3-5 days.

Part II : Determination effect of *relE* gene over-expression in *M. smegmatis* mc^{2}155 under stressful conditions

In order to investigate the effect of *relE* gene over-expression on the growth of *M. smegmatis* mc^{2}155, the replicative plasmid pMV261 carrying *M. tuberculosis relE* gene was electrotransformed into *M. smegmatis* mc^{2}155. For control, the parental pMV261 was used to transform. The obtained transformants were tested for their survival rates under various growth conditions, such as growth in acid medium, growth after expose to reactive oxygen and reductive nitrogen intermediates, growth under nutritional starvation and growth under high temperature.

3.5.19 Determination of growth rate in LB medium

A single colony of *M. smegmatis* mc^2155 was inoculated to 100 ml of LB broth containing 0.05% tween 80 whereas a single colony of test strain, *M. smegmatis* mc^2155 :: pMV261/*relE*, and control strain, *M. smegmatis* mc^2155 :: pMV261, were inoculated to 100 ml of LB broth containing 0.05% Tween 80 and 50 µg/ml of kanamycin in 250 ml flask. The cultures were incubated at 37 °C with a shaking speed of 200 rpm for 2 days. Cells were inoculated into 100 ml of LB broth containing 0.05% Tween 80 with or without 50 µg/ml of kanamycin to reach an initial OD_{600} at approximately 0.05. The cultures were then incubated at 37 °C and some aliquots of each culture were taken every 3 hours for 60 hours. The OD_{600} was measured and cell suspensions were serially diluted and plated on LB agar with or without 50 µg/ml of kanamycin for colony count.

3.5.20 Growth in acid environment

A single colony of test and control strains were grown in 100 ml LB broth pH 7.4 containing 0.05% tween 80 and 50 µg/ml of kanamycin whereas the parental strain was grown in 100 ml LB broth pH 7.4 containing 0.05% tween 80 at 37 °C with shaking at 200 rpm for 2 days. Cells were inoculated into 100 ml of LB broth containing 0.05% tween 80 with or without 50 µg/ml of kanamycin, whose pH adjusted to 5.5, 6.5 and 7.4 to reach an initial OD_{600} at 0.05. The cultures were incubated under the same condition as described above. Some aliquots of each culture were taken every 6 hours for 60 hours. The cell culture were serially diluted and plated on LB agar with or without 50 µg/ml of kanamycin for colony count. The survival rate of the transformants and wild type is determined comparing with their growth in the optimal pH.

3.5.21 Growth under reactive oxygen intermediates (ROI)

The transformants were cultivated in 100 ml of LB medium containing 0.05% tween 80 and 50 µg/ml of kanamycin at 37 °C with shaking at 200 rpm for 30 hours or at mid-log phase. Five millilitres of mid-log phase cultures were treated with hydrogen peroxide at final concentrations of 0, 5, 10 and 20 mM for 2 hours. The cultures were then serially diluted and plated on LB agar containing 50 µg/ml of kanamycin for CFU determination. Survival of each strain was expressed as the percentage of CFU obtained from treated cultures compared with those from untreated cultures.

3.5.22 Growth under the reactive nitrogen intermediates (RNI)

The transformants were cultivated in 100 ml of LB medium containing 0.05% tween 80 and 50 µg/ml of kanamycin at 37 °C with shaking at 200 rpm for 30 hours. Cells were

harvested by centrifugation at 5,000 rpm at 4 °C for 5 min and resuspended in 100 ml LB broth pH 5.2. Five milliliters of cell suspension were exposed to sodium nitrite at the final concentrations of 0, 5, 10, 15 and 20 mM. Then, the suspension was incubated at 37 °C for 0, 2, 4 and 6 h. Each suspensions were then serially diluted and plated on LB agar containing 50 μg/ml of kanamycin for CFU determination. Survival of each strain was expressed as the percentage of CFU obtained from treated cultures compared with those from untreated cultures.

3.5.23 Growth under nutritional starvation

Form the preliminary experiment showing that *M. smegmatis* could survive in sterile deionized water for at least 2 weeks, therefore, the starvation experiment was designed to used deoinized water as medium for *M. smegmatis*. The transformants were cultivated in 100 ml of LB broth containing 0.05% tween 80 and 50 μg/ml of kanamycin at 37 °C with shaking at 200 rpm for 30 h. Cells from 50 ml of mid-log phase cultures were harvested by centrifugation at 5,000 rpm at 4 °C for 5 min, washed with 50 ml deionized water and resuspened in 100 ml of deionized water. The cell suspension was inoculated into either 100 ml of LB or deionized water containing 0.05% tween 80 and 50 μg/ml of kanamycin to get an initial OD_{600} at 0.05. The cultures were incubated at 37 °C with shaking at 200 rpm. Some aliquots of each culture were taken every 6 hours for 60 hours. The cell cultures were serially diluted and plated on LB agar containing 50 μg/ml of kanamycin for CFU determination. Survival of each strain was expressed as the percentage of CFU obtained form cells grown in deionized water compared with those grown in the enriched medium.

3.5.24 Growth under high temperature

The transformants were cultivated in 100 ml of LB medium containing 0.05% tween 80 and 50 μg/ml of kanamycin at 37°C with shaking at 200 rpm for 30 hours. Five millilitres of mid-log phase cultures were exposed to temperature 40, 46 and 52 °C for 10 min, subsequently. The cultures were serially diluted and plated on LB agar containing 50 μg/ml of kanamycin for CFU determination. Survival of each cells was expressed as the percentage of CFU obtained form exposed cultures compared with unexposed cultures.

3.5.25 Data analysis

Experimental method used was a completely randomized design (CRD) as basic design with three replications. In order to determine the effect of *relE* on cell viability, the CFU count of test and control strains, that grown under various stressful conditions was compared and

summarized. Data were statistically analyzed by Duncan's new multiple range test (DMRT) with the Statistical Package for Social Sciences (SPSS) windows software version 12.0.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Construction of the recombinant plasmid pMV261 containing *relE* gene

The first objective of the thesis was to construct the recombinant plasmid pMV261 containing *M. tuberculosis* H37Rv *relE* gene. To achieve an aim, *relE* gene was amplified from *M. tuberculosis* H37Rv crude DNA and ligated with the TA cloning vector pDrive. The pDrive-*relE* was prepared for cloning into *E. coli*/*Mycobacterium* shuttle vector pMV261 by digestion with *Bam*HI and *Cla*I. The digested *relE* gene fragment was ligated into the digested plasmid pMV261. The recombinant plasmid, pMV261-*relE* was transformed into competent cells *E. coli* DH5α. Transformants were selected on kanamycin containing LB agar. After that, the recombinant plasmid pMV261-*relE* was used directly to transform into *M. smegmatis* mc^2155 by electrotransformation. Growth of transformants was studied under stressful conditions compared to the control strain.

4.1.1 PCR amplification of *M. tuberculosis* H37Rv *relE* gene

Primers were designed based on the published genome sequence of *M. tuberculosis* H37Rv (Cole et al., 1998). The forward primer, FB2866, was designed by adding *Bam*HI recognition site before the start codon of *relE* gene (5′-GC<u>GGATCC</u>ATGCCTTACACCGTCG-3′) and reverse primer, RC2866, was designed by adding *Cla*I recognition site after the stop codon of *relE* gene (5′-CG<u>ATCGAT</u>GGTGAGTTGCTATCGGCG-3′). Crude DNA of *M. tuberculosis* H37Rv was prepared and used as a template for PCR reaction. PCR reaction and condition were described in section 3.5.4. After amplification, the 287-bp amplified products were analyzed by 1% agarose gel electrophoresis (Fig. 4.1). No PCR product was found in the negative control using deionized water instead of crude DNA as a template (Fig. 4.1). Before ligation, the amplified *relE* was purified by using the QIAquick gel extraction kit and determined the concentration by 1% agarose gel electrophoresis compared with 100 bp ladder DNA marker. The concentration of the purified PCR product was approximately 20 ng/μl (data not shown).

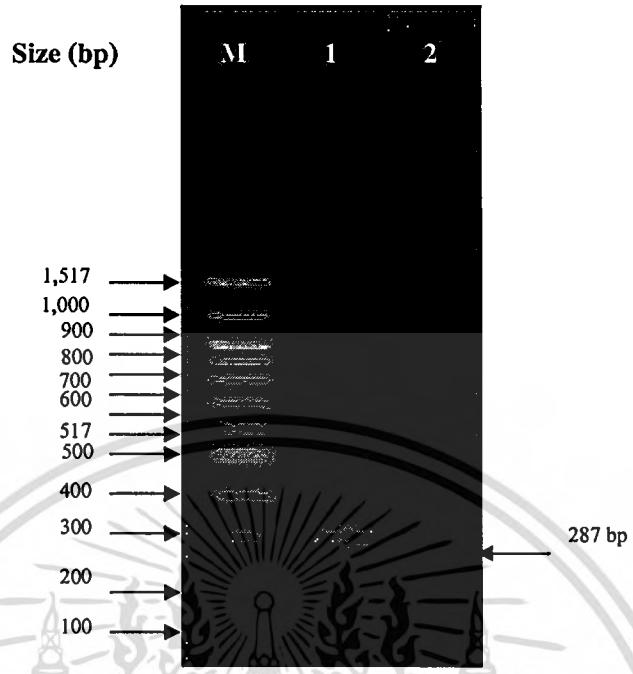


Figure 4.1 Agarose gel analysis of PCR product amplified by using crude DNA of *M. tuberculosis* H37Rv as a template (1) and negative control by using deionized water as a template (2). (M = 100 bp ladder DNA marker)

4.1.2 Cloning of *relE* PCR product into the TA cloning vector

The TA cloning vector, pDrive, was used for cloning PCR product in this thesis. Forty nanograms of the purified *M. tuberculosis* H37Rv *relE* gene was ligated to 50 ng of the plasmid pDrive. The recombinant plasmid pDrive-*relE* was transformed into the competent cell *E. coli* DH5α with high transformation efficiency of 10^6 CFU/μg DNA. Approximate 100 colonies were found on LB agar containing 50 μg/ml kanamycin, 80 μg/ml X-gal and 0.5 mM IPTG. About 20% of total colonies were white in color, suggesting the present of PCR product in these clones. Four white colonies were selected. They were grown in LB containing 50 μg/ml of kanamycin with shaking at 250 rpm at 37 °C for overnight. Cells were harvested by centrifugation of 12,000 rpm for 1 min and the plasmid DNA of the transformants were isolated.

4.1.3 Isolation of the recombinant plasmid pDrive-*relE* and restriction enzyme digestion

Plasmids DNA of four white transformants were isolated by QIAprep Spin Miniprep Kit and analyzed by 0.8% agarose gel electrophoresis as shown in Fig. 4.2. Only one dense DNA band of plasmid DNA was found in all isolates at the identical size. Therefore, the recombinant plasmid pDrive-*relE* of isolated 1.2 was selected to confirm the correct insertion of PCR product by restriction endonuclease digestion.

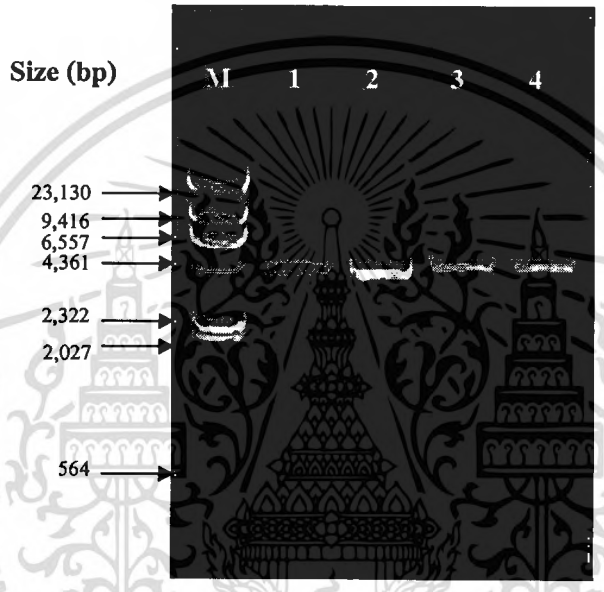


Figure 4.2 Analysis of the recombinant plasmid pDrive-*relE* clone 1.1-1.4 (lane 1-4) by 0.8% agarose gel electrophoresis. (M = λDNA/*Hin*d III fragments)

Plasmid DNA isolated from clone 1.2 was digested by restriction endonuclease *Eco*RI. Three digested bands in size of 94, 206, and 3,845 bp were detected on the 1.5% agarose gel (Fig. 4.3). Normally, *Eco*RI recognition site is located flanking the insertion site of the pDrive vector (Appendix A). In addition, one *Eco*RI recognition site is also found in *M. tuberculosis* H37Rv *relE* gene. Therefore, plasmid DNA isolated from clone 1.2 was the correct recombinant plasmid because it showed 3 bands of DNA fragments after *Eco*RI digestion.

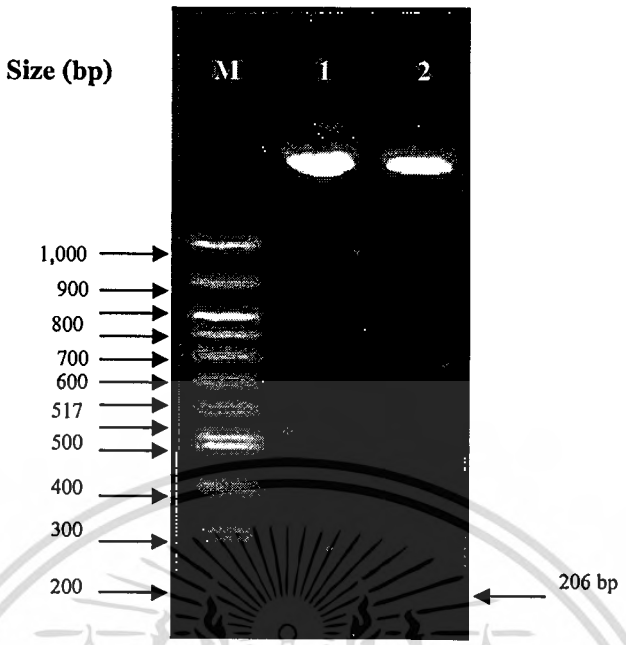


Figure 4.3 Recombinant plasmid analysis by digestion of plasmid pDrive-*relE* clone 1.2 with *Eco*RI (lane 1) compared with undigested plasmid pDrive-*relE* clone 1.2 (lane 2) in 1.5% agarose. (M = 100 bp ladder DNA marker)

The purified recombinant plasmid pDrive-*relE* isolated from clone 1.2 was confirmed for correct nucleotide sequence by submitted for DNA sequencing using T7 and SP6 as sequencing primers. Big-Dye™ terminator cycle sequencing ready reaction kit was used for the sequencing reaction with an ABI PRISM® 3700 DNA analyzer. The sequence chromatogram of recombinant plasmid pDrive-*relE* with T7 universal primer was shown in Fig. 4.4. The nucleotide sequence of 287 bp *relE* PCR product was shown in Fig. 4.5. The obtained nucleotide sequence showed 100% identity to the published sequence of *M. tuberculosis* H37Rv *relE* gene (Cole *et al.*, 1998). In addition, the correct *Bam*HI and *Cla*I recognition sequences were found (Fig. 4.5). From this result, it could be summarized that plasmid pDrive-*relE* isolated from clone 1.2 was the recombinant plasmid pDrive carrying the *M. tuberculosis* H37Rv *relE* gene. Therefore, this plasmid was selected for further subcloning the *relE* gene into the vector pMV261.

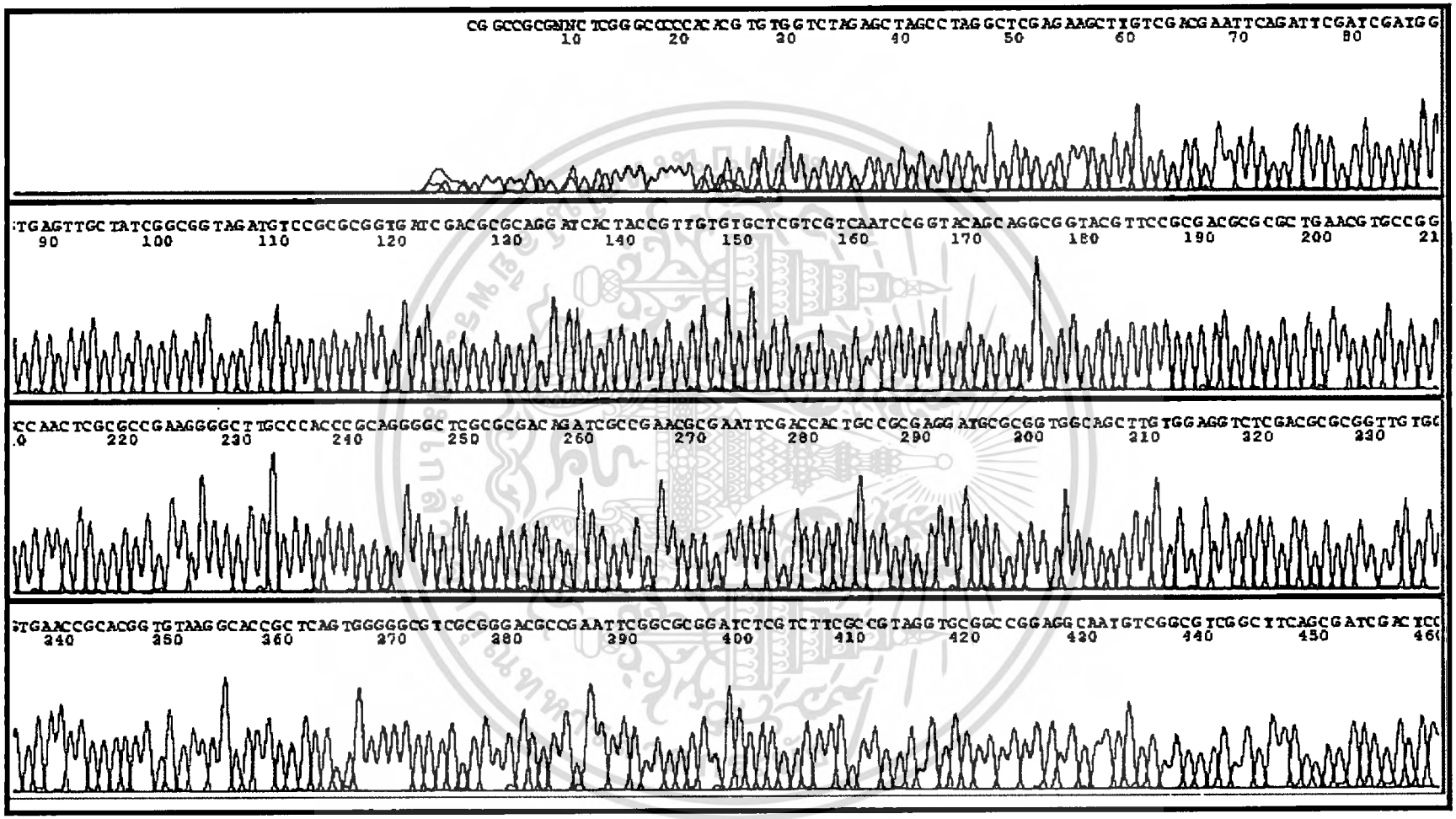


Figure 4.4 Sequencing chromatogram of plasmid pDrive-*relE* by using ABI PRISM^{R} 3700 DNA analyzer from BigDye terminator reactions with universal T7 primer

*Bam*HI **Start codon**

1	GCGGATCCAT GCCTTACACC GTGCGGTTCA CCACAACCGC GCGTCAGACC 50
51	TCCACAAGCT GCCACCGCGC ATCCTCGCGG CAGTGGTCGA ATTCGCGTTC 100
101	GGCGATCTGT CGCGCGAGCC CCTGCGGGTG GGCAAGCCCC TTCGGCGCGA 150
151	GTTGGCCGGC ACGTTCAGCG CGCGTCGCGG AACGTACCGC CTGCTCTACC 200
201	GGATTGACGA CGACACACAA CGGTAGTGAT CCTGCGCGTC GATCACCGCG 250
251	CGGACATCTA CCGCCGATAG CAACTCACCA TCGATCG

Stop codon *Cla*I

Figure 4.5 Nucleotide sequence of pDrive-*relE* showing the 287-bp *M. tuberculosis* H37Rv *relE* gene. Primers FB2866 and RC2866 were shown in square boxes. Recognition sites of *Bam*HI and *Cla*I were shown in red and blue, respectively.

4.1.4 Subcloning of *relE* to *E. coli* / *Mycobacterium* shuttle vector pMV261

In order to study the effect of *M. tuberculosis relE* gene on stress response the *relE* gene was subcloned into the replicative vector pMV261 and overexpressed in *M. smegmatis* mc^2155. pMV261 is a shuttle vector which can replicate itself in *E. coli* and mycobacteria as an extrachromosomal low copy-number plasmid, one to five copies per cell. This vector carries the *M. bovis hsp65* promoter upstream from the multiple cloning sites, allowing expression of promoterless cloned genes (Stover *et al.*, 1991). The multiple cloning sites of pMV261 contain a number of different restriction enzyme digestion sites including recognition sites of *Bam*HI and *Cla*I (Appendix A). Thus, the digested *Bam*HI-*Cla*I pMV261 vector can be used directly for cloning *relE* fragment from the pDrive-*relE* digested with *Bam*HI and *Cla*I. Schematic diagram of subcloning of *relE* gene in shuttle vector pMV261 was shown in Fig. 4.6. In this study, plasmid pMV261 was prepared by using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and analyzed by 0.8% agarose gel electrophoresis. A clearly band of approximately 4,500 bp in size of plasmid pMV261 was observed in 0.8% agarose gel (Fig. 4.7). The concentration of plasmid pMV261 was about 50 ng/µl. For ligation reaction, both isolated plasmid pMV261 and recombinant plasmid pDrive-*relE* were double digested with restriction enzyme *Bam*HI and *Cla*I following the protocol in table 3.4. The digested products were analyzed by agarose gel electrophoresis. It was shown that an upper band of the *Bam*HI-*Cla*I digested

pMV261, in size of 4,480 bp was found in 0.8% agarose gel when compared with the size of uncut plasmid pMV261 (Fig. 4.8). Whereas *Bam*HI and *Cla*I digested pDrive-*relE* gave the two DNA fragment bands, upper band of a 3,860 bp plasmid pDrive and lower band of a 277 bp *relE* fragment in 1% agarose gel (Fig. 4.9).

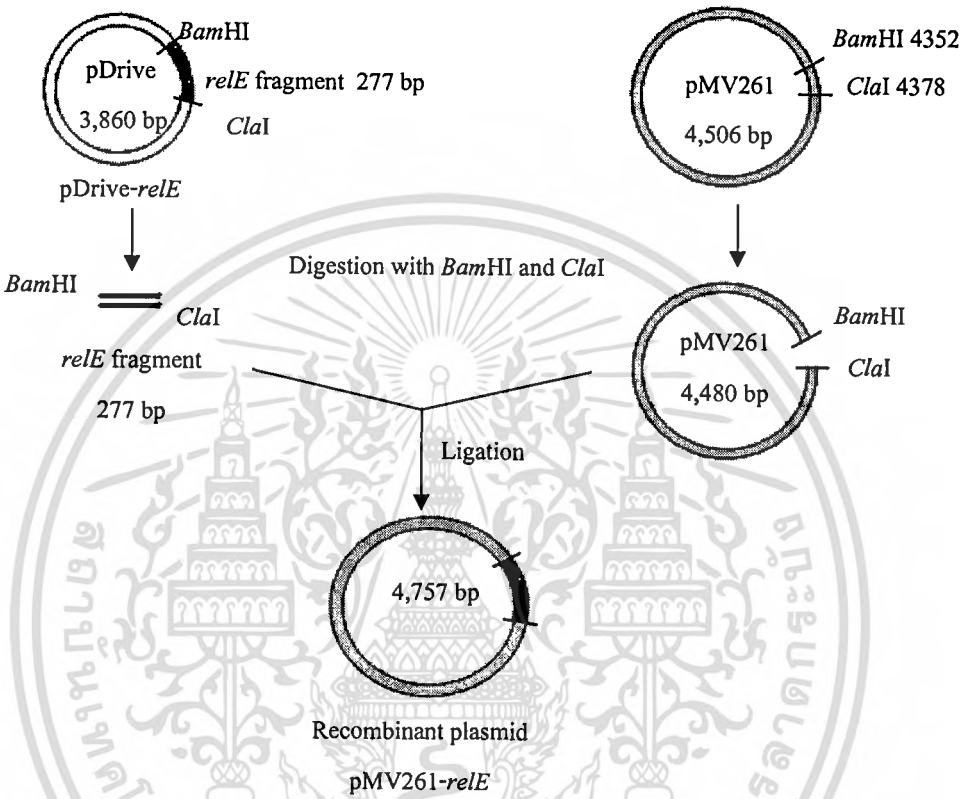


Figure 4.6 Schematic diagram of subcloning of *relE* gene in the shuttle vector pMV261.

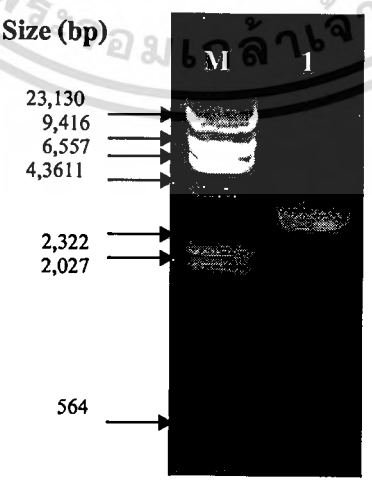


Figure 4.7 Plasmid pMV261 analyzed by 0.8% agarose gel. (M = λDNA/*Hin*d III fragments)

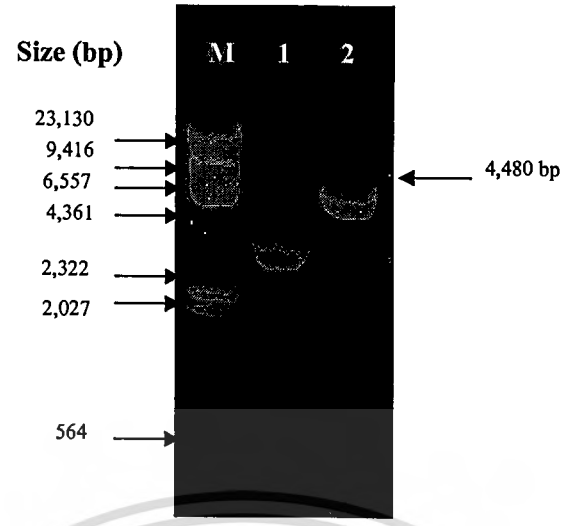


Figure 4.8 The *Bam*HI-*Cla*I digested plasmid pMV261 (lane 2) compared with uncut plasmid pMV261 (lane1) analyzed by 0.8% agarose gel. (M = λDNA/*Hin*d III fragments)

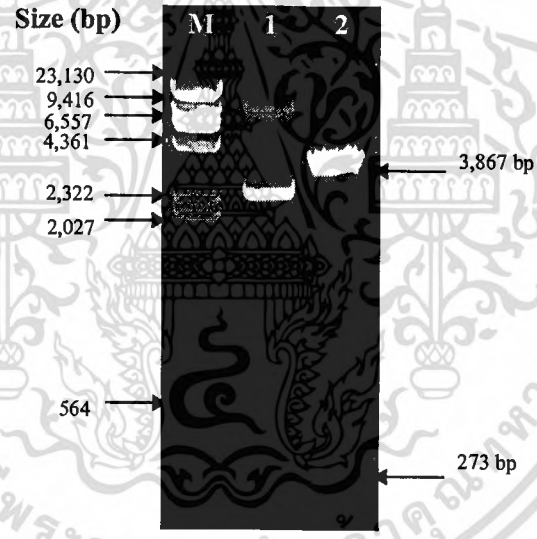


Figure 4.9 The *Bam*HI-*Cla*I digestion of plasmid pDrive-*relE* (lane 2) compared with uncut plasmid pDrive-*relE* (lane 1) analyzed by 1% agarose gel. (M = λDNA/*Hin*d III fragments)

Both DNA fragments of *Bam*HI-*Cla*I digested pMV261 and *relE* gene were purified by using QIAquick gel extraction kit. The concentrations of purified *Bam*HI-*Cla*I digested pMV261 and purified *relE* gene fragments were 50 ng/μl. The purified DNA fragment of *relE* gene, in size of 273 bp, was ligated to the 4,480-bp *Bam*HI-*Cla*I digested plasmid pMV261 at an optimum ratio of 4 : 3. The ligation product was chemically transformed to the competent

cells *E. coli* DH5α with a transformation efficiency of 1.45 x 10^6 CFU/μg. Approximate 100 colonies per plate were found on LB plate containing 50 μg/ml of kanamycin.

4.1.5 Analysis of pMV261-*relE* clones

Six colonies grown on kanamycin containing LB agar were picked up and cultured in LB broth containing 50 μg/ml of kanamycin. The correct insertion of *relE* gene was firstly checked by PCR. Crude DNA of transformants was isolated according to method in section 3.5.2 and used as a template in a PCR reaction. The published nucleotide sequence of pMV261 (Stover *et al.*, 1991) was used to create forward primer at 30 bp upstream of *Bam*HI site, called pMV261-FBam, and a reverse primer at 70 bp downstream of *Cla*I site, called pMV261-RCla. By PCR reaction, a PCR product of 375 bp was amplified, consisting of 275 bp of the *relE* gene and 100 bp of pMV261, in a positive clone. The PCR products were analyzed by 1% agarose gel electrophoresis. Results revealed a PCR product with an expected size of 375 bp from transformant clone 8.12 as shown in Fig. 4.10.

Figure 4.10 PCR product from a PCR reaction of crude DNA isolated from transformant clone 8.8-8.13 (lane 1-7) using primer pMV261-FBam and pMV261-RCla. (M = λDNA/*Hin*d III fragments)

Further confirmation was carried out by restriction endonuclease digestion. Plasmid DNA from clone 8.12 was isolated and double digested with *Eco*RI and *Xba*I. The recognition site of *Eco*RI was found only one position in *relE*, whereas one recognition site of *Xba*I was found in pMV261. It was found that two DNA fragments in size of approximate 3,500

and 700 bp were detected by 1% agarose gel electrophoresis (Fig. 4.11). This result confirmed the result of PCR that plasmid DNA isolated from clone 8.12 was the recombinant plasmid of pMV261 and *relE*, designated as pMV261-*relE*. The recombinant plasmid pMV261-*relE* was isolated and sequenced with pMV261-FBam and pMV261-RCla primers. Big-Dye™ terminator cycle sequencing ready reaction kit was used for the sequencing reaction with an ABI PRISM® 3700 DNA analyzer. The chromatogram of the recombinant plasmid pMV261-*relE* was presented in Fig. 4.12. Nucleotide sequence analysis revealed the presence of *relE* sequence connected to plasmid pMV261 sequence. Moreover, the nucleotide sequence of pMV261-*relE* consisted of recognition sites of *Bam*HI, *Eco*RI and *Cla*I, including nucleotide sequences of pMV261-FBam and pMV261-RCla primers (Fig 4.13). The result of PCR reaction together with enzyme digestion and nucleotide sequencing indicated that *M. tuberculosis* H37Rv *relE* was successfully cloned into pMV261 resulted in plasmid pMV261-*relE*.

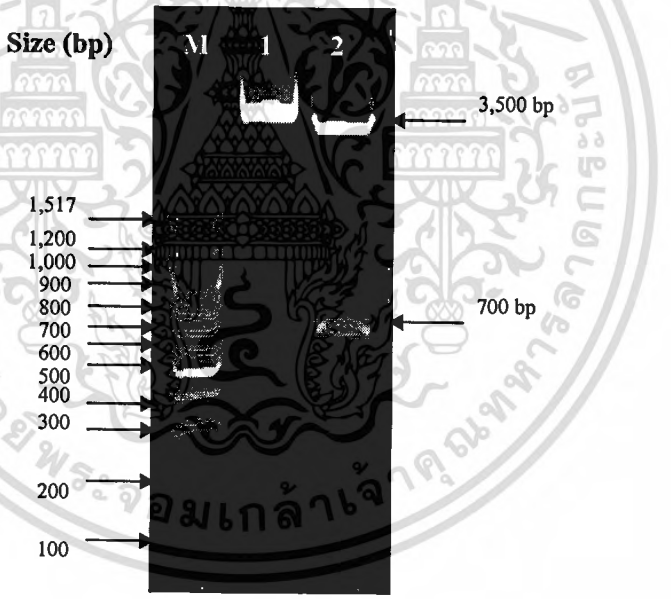


Figure 4.11 Recombinant plasmid analysis by digestion of plasmid pMV261-*relE* clone 8.12 with *Eco*RI and *Xba*I (lane 2) compared with undigested plasmid pMV261-*relE* clone 8.12 (lane 1) in 1% agarose gel. (M = 100 bp ladder DNA marker)

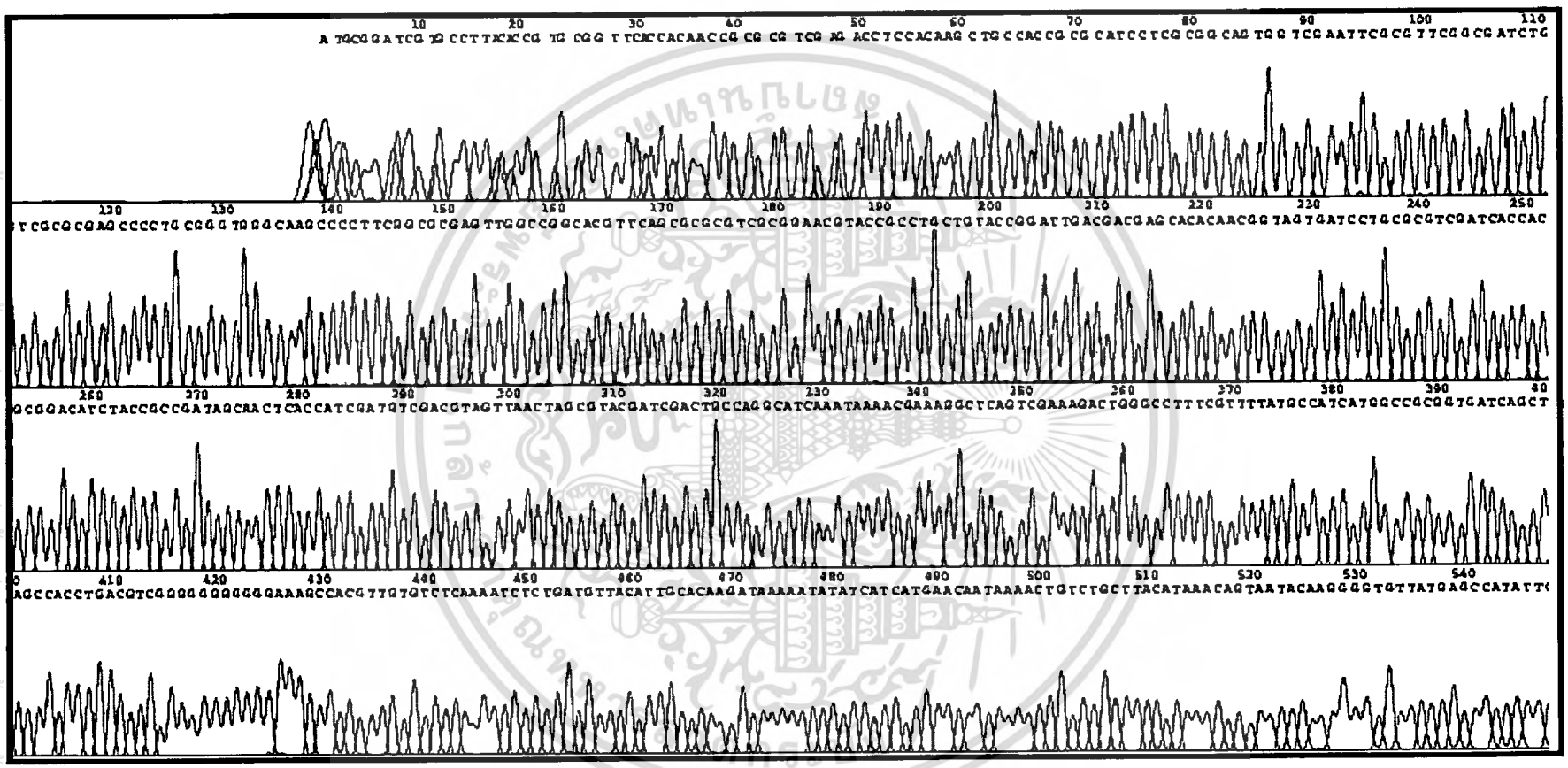


Figure 4.12 Sequencing chromatogram of plasmid pMV261-*relE* by using ABI PRISM^{R} 3700 DNA analyzer from BigDye terminator reaction with pMV261-FBam primer

pMV261-FBam primer

1	AGGAATCACT TCGCAATGGC CAAGACAATT GC**GGATCC**GT GCCTTACACC 50
51	**ATG**CGGTTCA CCACAACCGC GCGTCGAGAC CTCCACAAGC TGCCACCGCG 100
101	CATCCTCGCG GCAGTGGTCG AATTCGCGTT CGGCGATCTG TCGCGCGAGC 150
151	CCCTGCGGGT GGGCAAGCCC CTTCGGCGCG AGTTGGCCGG CACGTTCAGC 200
201	GCGCGTCGCG GAACGTACCG CCTGCTGTAC CGGATTGACG ACGAGCACAC 250
251	AACGGTAG**TG A**TCCTGCGCG TCGATCACCG CGCGGACATC TACCGCCGAT 300
301	AGCAACTCAC CATCGATGTC GACGTAGTTA ACTAGCGTAC GATCGACTGC 350
351	CAGGCATCAA ATAAAACGAA AGGCTCAGTC GAAAGA

pMV261-RCla primer

Figure 4.13 Nucleotide sequence of plasmid pMV261-*relE* showed 273 bp of *M. tuberculosis* H37Rv *relE* gene with pMV261-FBam and pMV261-RCla primer in square boxes including recognition sites of *Bam*HI and *Cla*I shown in red and blue letters, respectively. Start codon (ATG) and stop codon (TGA) were shown in underline letters.

4.1.6 Electrotransformation of pMV261-*relE* into *M. smegmatis* mc^{2}155

Approximate 0.1 μg of recombinant plasmid pMV261-*relE* was transformed into the *M. smegmatis* mc^{2}155 by electroporation as described in the methods section 3.5.18. *M. smegmatis* was a generally non-pathogenic mycobacterium. The mutant of *M. smegmatis*, strain mc^{2}155, is more efficiently transformed using electroporation than the parental strain and thus is valuable in analysis of mycobacterial gene function, expression and replication (Zainuddin *et al.*, 1988). After electroporation, cells were plated on LB agar containing 50 μg/ml of kanamycin. A total of 100 colonies were found and a single colony was picked to study the effect of *relE* gene when over-expressed in *M. smegmatis* mc^{2}155 under stressful conditions.

4.2 Effect of *relE* gene over-expression on growth of *M. smegmatis* mc^{2}155 under stressful conditions

Most TA systems, including *relBE* modules in mycobacteria, contributed to the control of macromolecule synthesis in mycobacterial cells. The toxin RelE was a global inhibitor of translation that neutralized by antitoxin RelB by a directional protein-protein interaction under normal growth conditions (Christensen and Gerdes, 2004). In the stressful conditions, degradation of antitoxin resulted in the release of toxin allowing toxin to exert its effect on the cell (Korch *et al.*, 2009). The over-expression of *relE* gene confers severe inhibition of translation, inhibition of cell growth, and reduction in the number of colony-forming cells (Pedersen *et al.*, 2003) so toxin could help identify targets for development of wide-spectrum antibiotics (Gupta, 2008). As mentioned above, the expression of toxin *relE* gene was regulated by stress conditions such as amino acid starvation, thymine starvation (Sat *et al.*, 2003), UV irradiation and oxidative stress (Hazan *et al.*, 2004). Therefore, this part of thesis was to investigate the effect of *relE* gene on growth of *M. smegmatis* mc^{2}155 under various stressful conditions such as in acid environment, under reactive oxygen or nitrogen intermediate, under nutritional starvation and under high temperature. To determine the kinetic of *M. smegmatis* mc^{2}155 growth inhibition conferred by *relE* gene, *M. smegmatis* mc^{2}155 containing the parental pMV261 plasmid was used as a control. Their growth patterns were examined by counting cell number in colony forming units (CFU).

4.2.1 Growth of the *relE* toxin-producing strain under normal condition

M. smegmatis mc^{2}155::pMV261-*relE* (test strain) and *M. smegmatis* mc^{2}155::pMV261 (control strain) were grown in LB broth (pH 7.4) containing 50 μg/ml of kanamycin and 0.05% Tween 80 with a shaking speed of 200 rpm at 37 °C for 60 h. Cell number was determined by counting colony forming units. No significant difference (p>0.05) of growth rate was determined among both strains during 60 h of cultivation (Fig. 4.14). After inoculation, both cultures were slightly grown during lag phase period from 0-12 h of cultivation. They reached the mid-logarithmic phase within approximately 30-36 h of cultivation. After 48 h, they reached the stationary phase. Similarity of growth rate of both strains indicated that under normal condition over-expression of toxin RelE did not affect the bacterial growth.

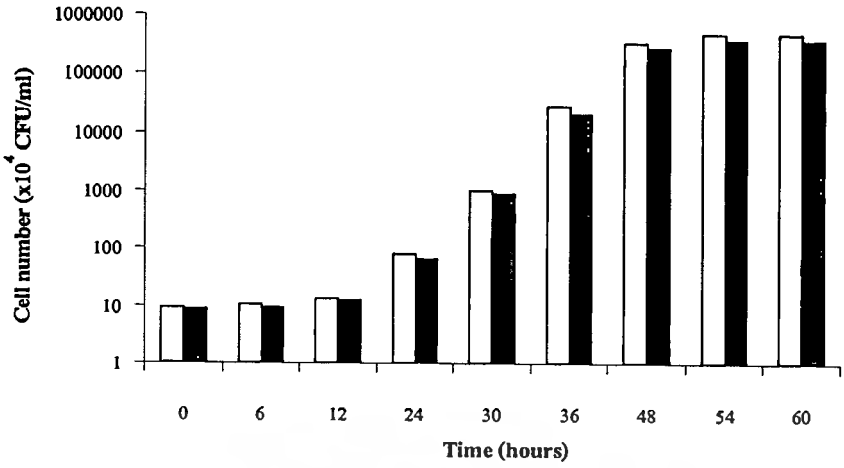


Figure 4.14 Growth of strain *M. smegmatis* mc^{2}155::pMV261 (white) and *M. smegmatis* mc^{2}155::pMV261-*relE* (black) in LB broth pH 7.4 containing 0.05% Tween 80 and 50 µg/ml of kanamycin pH 7.4 monitored by colony forming units.

4.2.2 Growth in acid environment

An acidic environment is one of the most stressful conditions encountered by bacterial cells. To analyze the effect of *relE* gene expression in a qualitative manner, the growth rate of test and control cells was estimated in LB broth. Growth of *M. smegmatis* mc^{2}155 containing *relE* gene and control strain in LB broth at pH 5.5 and 6.5 were examined compared with growth under normal condition (pH 7.4). This pH range was chosen because it mimics the pH range of macrophage phagosome (pH 6.1-6.5) where *M. tuberculosis* was dormancy. It should ordinarily not be harmful to the cells (Sturgill-Koszycki *et al.*, 1994; Oh and Straubinger, 1996). Results revealed no significant difference (p>0.05) of growth was found between both strains in each pH condition (Fig 4.15). In addition, cell number of both strains was not different when grown in each pH condition (5.5 or 6.5 and 7.4). Generally, mycobacteria are likely to encounter both acidic and mildly acidic pH in the host environment (Sturgill-Koszycki *et al.*, 1994; Oh and Straubinger, 1996; Iivanainen *et al.*, 1999). Portaels and Pattyn (1982) reported that *M. smegmatis* was capable of growth over a wide pH range providing an optimum growth at pH between 5.0 to 7.4, and a partial growth at pH 4.6. The effect of acidic stress with *relE* gene expression was related to internal pH rather than external pH in cells. In order to survive in acidic pH, mycobacteria adapted an intercellular pH close to range 6.1-7.2 (Rao *et al.*, 2001). Zhang and colleague (1999) reported that at an external pH of 5.0, the internal pH of *M. tuberculosis* H37Ra was close to 7.0, suggesting the pH homeostasis adaptation of *M. tuberculosis*. Thus, the constant internal pH under external acidic pH at 6.5 and 5.5 did not affected *relE* gene expression. Multiple systems

were utilized to maintain a neutral internal pH for enzyme and protein functions. These include transport system which exchanges protons for cations, system which transports protons out of cells in association with ATP hydrolysis, and the production of cytoplasmic macromolecules which function as internal buffers (Piddington *et al.*, 2000).

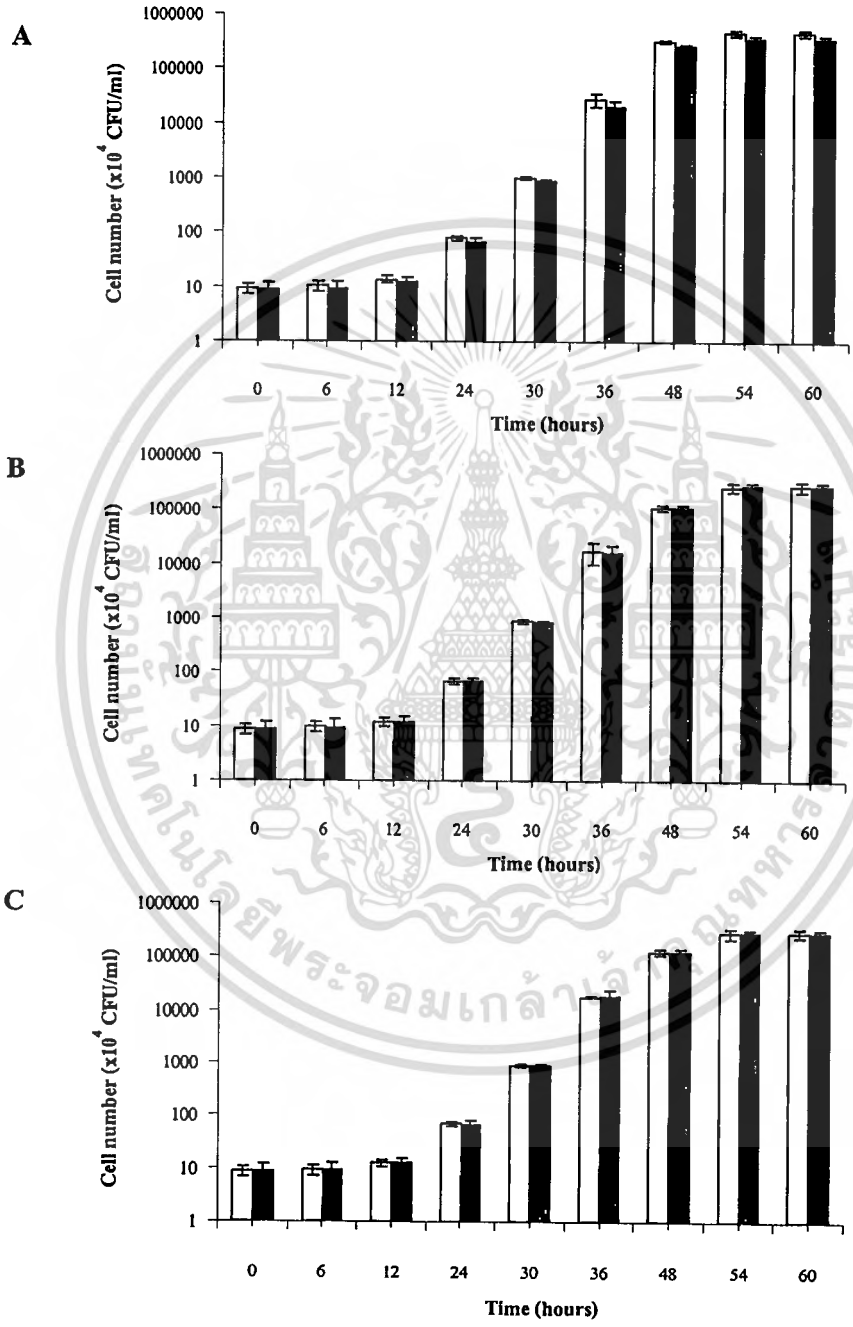


Figure 4.15 Growth of strain *M. smegmatis* mc^{2}155::pMV261 (white) and *M. smegmatis* mc^{2}155::pMV261-*relE* (black) in the acidic LB broth pH 5.5 (B) and 6.5 (C) compared with growth under optimal condition (pH 7.4) (A).

4.2.3 Growth under reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI)

In view of the response of *relE* gene to reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), viable plate counts were obtained after exposure of *M. smegmatis* mc^{2}155 containing *relE* gene and control strain to various concentrations of either hydrogen peroxide or acidified sodium nitrite as representative of ROI and RNI, respectively. To determine whether hydrogen peroxide contributed to the *relE* gene expression at the mid-log phase, the survival rate of *M. smegmatis* mc^{2}155 containing *relE* gene was measured and compared with control strain after exposure to 0, 5, 10 and 20 mM of hydrogen peroxide for 2 h. Three replicates were done and CFU were counted to calculate the percentage of survival rate for each strain or condition. It was found that growth of the *M. smegmatis* mc^{2}155 containing *relE* gene was significantly (p<0.05) inhibited in the presence of hydrogen peroxide as shown in figure 4.16. The survival rate of *M. smegmatis* mc^{2}155 containing *relE* gene was rapidly decreased as 70%, 10% and 1% of survival rate according to the increasing concentrations of hydrogen peroxide at 5, 10 and 20 mM, respectively. Whereas, the growth rate of control strain was slightly decreased as 85%, 70% and 56% of survival rate according to the increasing concentration at 5, 10 and 20 mM of hydrogen peroxide, respectively. In addition, survival rate of *M. smegmatis* mc^{2}155 containing *relE* gene and control strain was significantly (p<0.05) different when cells were grown in 10 and 20 mM of hydrogen peroxide. The result indicated that under higher hydrogen peroxide concentration, RelE promoted cell death in *M. smegmatis* resulting in the lower survival rate.

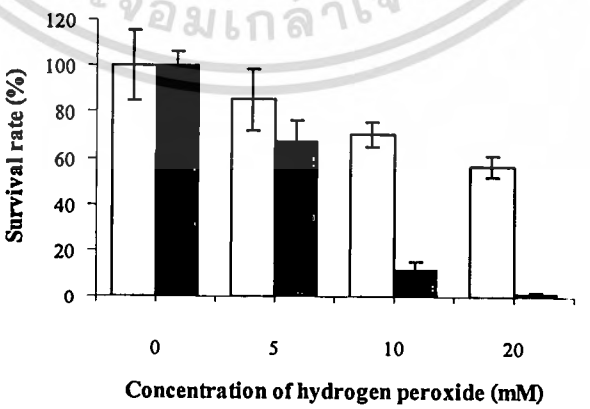


Figure 4.16 Survival rate of strains *M. smegmatis* mc^{2}155::pMV261 (white) and *M. smegmatis* mc^{2}155::pMV261-*relE* (black) after exposure to 0, 5, 10 and 20 mM of hydrogen peroxide for 2 h. Survival rate was significantly different at 10 and 20 mM (p<0.05).

To study the response of *relE* gene to various RNI concentrations, survival rate of both strains was measured and compared after exposure to 0, 5, 10, 15 and 20 mM of sodium nitrite for 0, 2, 4 and 6 h. It was found that the survival rate was gradually decreased in both strains when exposed cells to higher concentrations as shown in Fig. 4.17. Moreover, incubation time of exposure affected the survival rate of cells. However, the survival rate of *M. smegmatis* mc^2155 containing *relE* gene was similar to that of the control strain even when increased the incubation time of exposure. In addition, no significant difference ($p>0.05$) of survival rate was determined among both strains. It demonstrated that the stress response by RNI was not affected to the *M. smegmatis* mc^2155 containing *relE* gene.

ROI and RNI were the most effective antimycobacterial molecules generated by the host during infection which kill bacteria by damaging macromolecules such as bacterial DNA (Colangeli *et al.*, 2008). Generally, the immune system of human cells was promoted after inhalation of an infectious aerosol. This process leads to impeded replication of *M. tuberculosis* in alveolar macrophages (Voskuil *et al.*, 2003). Macrophages play an important role in the first and essential line of defense against mycobacterial disease (Edwards and Krikpatrick, 1986). Several observations about the antimicrobial functions of macrophages have emphasized their ability to produce ROI, RNI and various cytokines in rodent immune systems (Nozaki *et al.*, 1997). ROI, such as hydrogen peroxide, eliminates proteins through oxidation (Nishiyama *et al.*, 2001). The disinfection mechanism of hydrogen peroxide is based on the release of free oxygen radicals. Free radicals have both oxidizing and disinfecting abilities. Thus, the higher concentrations of hydrogen peroxide cause a pleiotropic reaction, including an oxidative stress response (Akaki *et al.*, 1997). In contrast, RNI contribute to control of microbial pathogens is unclear. Considering only proteins, sodium nitrite can nitrosylate cysteine sulfhydryls and heme prosthetic groups, disrupt iron–sulfur clusters and inactivate tyrosyl radicals (St. John *et al.*, 2001). The LB both for sodium nitrite experiment was adjusted to pH 5.5 because this appropriated the hydrogen ion concentration in the phagosome of activated macrophages. The acidity of the phagosome is likely to be critical to the antibacterial action of RNI (St. John *et al.*, 2001). The reasons why RNI did not affected all growth conditions are unexplained. It is possible that (i) RelE toxin requires additional intracellular components that trigger its effect and is present only in response to some stress conditions, and that (ii) there are antitoxin-like proteins in *M. smegmatis*, which could neutralize the effect of RelE toxin in most conditions, regardless of that there is no *relBE* homologue found in this organism.

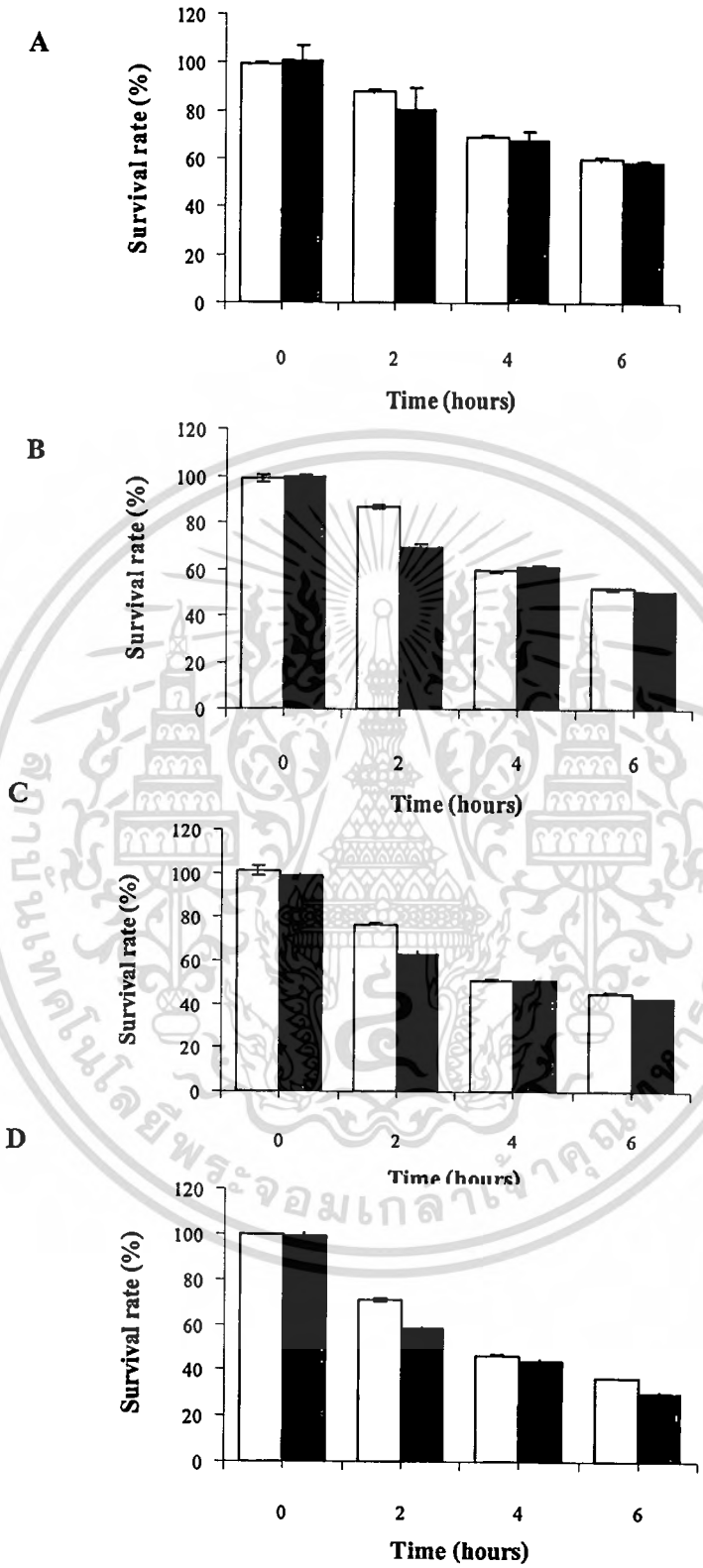


Figure 4.17 Survival rate of strains *M. smegmatis* mc^{2}155::pMV261 (white) and *M. smegmatis* mc^{2}155::pMV261-*relE* (black) after exposure to 5 (A), 10 (B), 15 (C) and 20 (D) mM of acidified $NaNO_2$ for 2, 4 and 6 hours.

4.2.4 Growth under nutritional starvation

Under various stress from host defense systems, nutritional starvation was one of the important stress. To investigate the response of *relE* to the nutritional starvation, the growth rate of *M. smegmatis* mc^{2}155::pMV261-*relE* and control strain was determined by counting CFU from cultures incubated in deionized water and grown in enrich medium (LB broth) for 1 week. It was found that cell number of control cells incubated in deionized water was slightly decrease everyday and still remained at 10^4 CFU/ml at all time period (Fig. 4.18A). In contrast to *M. smegmatis* mc^{2}155::pMV261-*relE*, cell number of test cells was rapidly decreased until they died after 6 to 7 day of incubation (Fig. 4.18A). Growth rate of test cells was significantly different ($p<0.05$) from the control cells after incubated cells in deionized water at the first day. Growth rates of control and test cells were not significantly different ($p>0.05$) when cells were grown in LB broth as shown in Fig. 4.19B. Growth rate of test and control cells were in agreement with the previous experiment (4.2.1) that middle period of log phase was between 30-36 h. In addition, cell number of both strains was constant at 10^9 CFU/ml at stationary stage after grown in LB for 3 days.

During the latent stage, *M. tuberculosis* is exposed to the low or restricted nutrient concentrations in the host and is able to survive under these conditions for long periods of time (Betts *et al.*, 2002). Several observations showed that the growth pattern between latency and *in vitro* starved cultures of various species of mycobacteria were similarity (Gupta *et al.*, 2008). The growth pattern of *M. tuberculosis* cultured in distilled water displayed ability to survive for extended periods in a non-growing state and regained growing when added to nutrient rich medium even after 2 year starvation period (Nyka, 1974). It was related to growth pattern of control cells cultured in distilled water that showed these same properties to survive in a non-growing state during the stress of nutritional starvation. In contrast, growth rate of test cells was rapidly decreased until cells were completely dead. This phenomenon was due to the over-expression of *relE* gene that was previously shown to promote a reversible cell cycle arrest program under condition of starvation (Pedersen *et al.*, 2003). Over-expression of *relE* gene reduced the levels of translation through the cleavage of translating mRNAs in a sequence specific manner with a preference for stop codons, codons adjacent to the start codon and codons with G or C in the third position (Korch *et al.*, 2009).

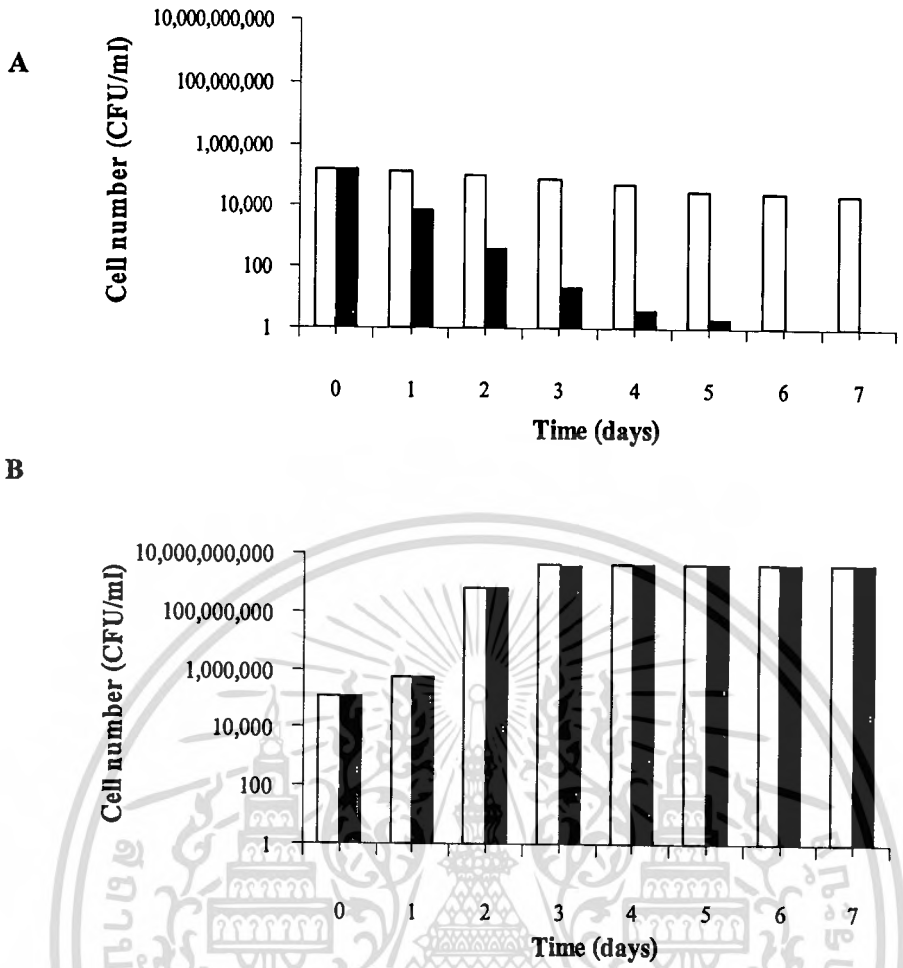


Figure 4.18 Viable cell count of strains *M. smegmatis* mc^{2}155::pMV261 (white) and *M. smegmatis* mc^{2}155::pMV261-*relE* (black) grown under starvation condition in deionized water (A) compared with growth in LB broth (B).

4.2.5 Growth under high temperature

To determine whether heat stress was contributed to the *relE* gene expression at the mid-log phase, the survival rates of test cells were measured and compared with control cells after exposure to 37, 42, 47 and 52 ºC for 0, 20, 40 and 60 minutes. It was found that when the temperature increased, lost of CFU after exposed to high temperature of both strains was increased and resulting in reduction of survival rate (Fig. 4.19). Moreover, increasing of exposure time supported more decreasing of survival rate. At the highest temperature in this experiment, 52 ºC, survival rate of test strain was rapidly reduced than that in another temperature from 100 to 40, 16 and 1 after exposed for 20, 40 and 60 minutes, respectively (Fig. 4.20C). Similar to the survival rate of control strain was reduced from 100 to 45, 19 and 3 after exposed for 20, 40 and 60 minutes, respectively (Fig. 4.20C). It was shown that control cell was more slightly tolerant with

heat response than test cell. However, no significant difference (p>0.05) was observed for the survival rate in all tested conditions.

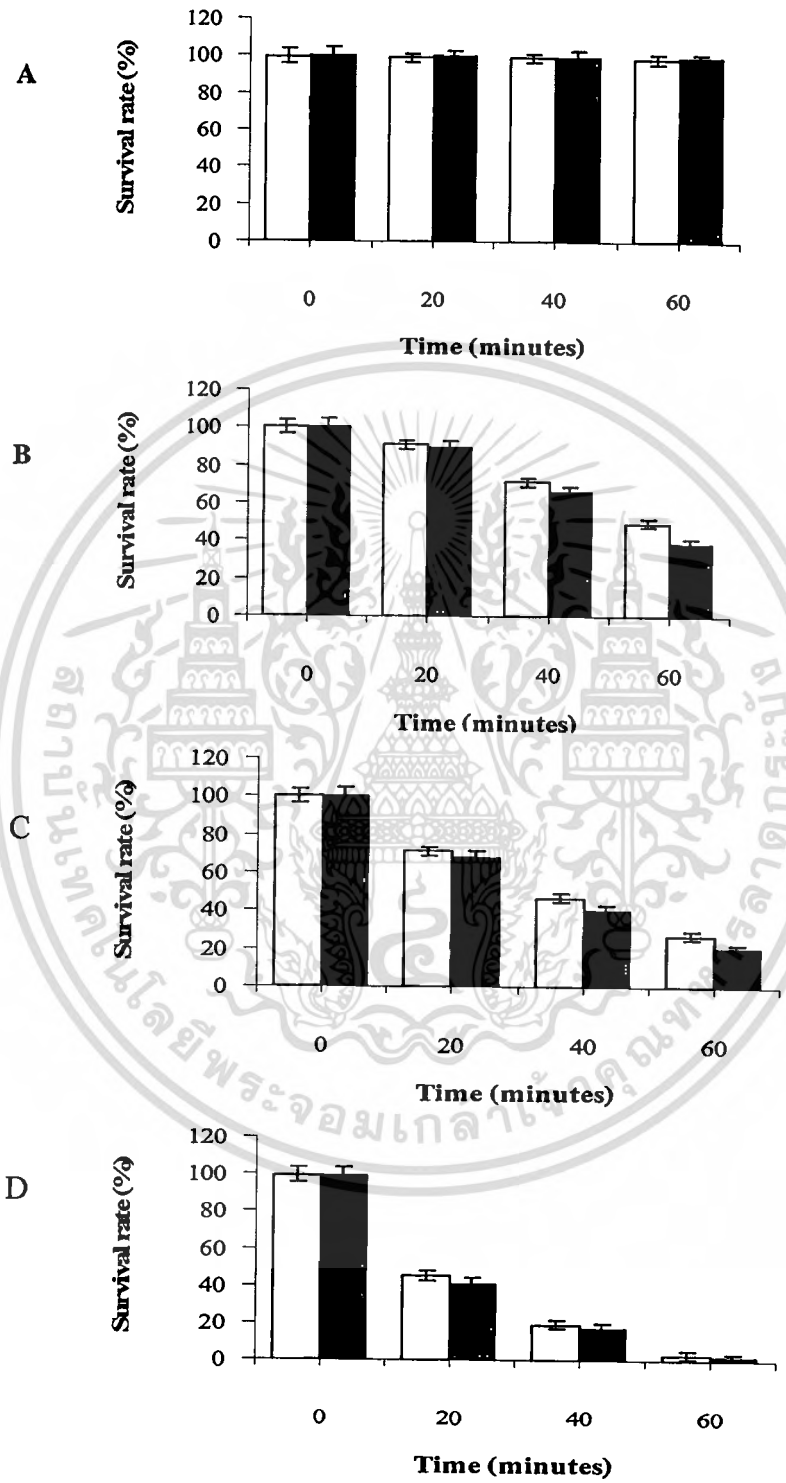


Figure 4.19 Survival rate of strains *M. smegmatis* mc^{2}155::pMV261-*relE* (white) and *M. smegmatis* mc^{2}155::pMV261 (black) after exposure to high temperature at 37°C (A), 42 °C (B), 47 °C (C) and 52 °C (D) for 20, 40, 60 min.

The stress response of *M. tuberculosis* after exposed to high temperature has been studied in several considerable details. In most case, it was indicated that the heat stress response was an adaptive pathway involved in the survival of bacteria that are exposed to increased ambient temperature (Patel *et al.*, 1991). It was characterized by genome-wide transcriptional changes and resulting in the induction of over 100 genes (Stewart *et al.*, 2002). Anyway, the result from this study indicated that elevated temperature affected to the survival of mycobacteria but was not involved in survival rate of *relE* overexpressed mycobacteria.

CHAPTER 5

CONCLUSIONS

This present study aims to determine the effect of *M. tuberculosis* H37Rv *relE* gene over-expression in *M. smegmatis* mc^2155 under various stressful conditions. For construction of the recombinant plasmid pMV261 containing *M. tuberculosis* H37Rv *relE* gene, 287 bp of *M. tuberculosis* H37Rv *relE* gene was amplified from *M. tuberculosis* H37Rv crude DNA by using FB2866 and RC2866 primers in a PCR reaction. The purified PCR product was ligated with TA cloning vector pDrive and the pDrive-*relE* was transformed into the competent cell *E. coli* DH5α. Plasmid DNA isolated from clone 1.2 was the correct recombinant plasmid pDrive-*relE* checked by *Eco*RI endonuclease digestion. Three bands of DNA fragments were found in expected size as 3,845 bp of pDrive and 206 bp with 94 bp of *relE* gene. Further confirmation was carried out by nucleotide sequencing with T7 and SP6 universal primers. The nucleotide sequence reported here showed 100% similarity to the sequence of *M. tuberculosis* H37Rv *relE* gene reported earlier. Therefore, plasmid pDrive-*relE* was selected to subclone *relE* gene to *E. coli*/*M. tuberculosis* shuttle vector pMV261 and transformed into the competent cell *E. coli* DH5α. The correct insertion of *relE* gene in plasmid pMV261 was firstly checked by using pMV261-FBam and pMV261-RCla primers in a PCR reaction. PCR products with expected size of 375 bp, 275 bp of *relE* gene and 100 bp of pMV261, were found when using crude DNA of transformant clone 8.12 as a template. The plasmid isolated from transformant clone 8.12 was confirmed by double restriction digestion with *Eco*RI and *Xba*I. It was found that two DNA fragments in expected size of approximate by 3,500 and 700 bp were detected. After that, the recombinant plasmid pMV261-*relE* was sequenced with pMV261-FBam and pMV261-RCla primers that revealed the presence of *relE* sequence connected to plasmid pMV261 sequence. Therefore, the recombinant plasmid pMV261-*relE* was used directly to transform into *M. smegmatis* mc^2155 by electrotransformation. Growth of transformant, test strain, was studied under stressful conditions compared to control strain. To determine the kinetic of *M. smegmatis* mc^2155 growth inhibition conferred by *relE* gene, *M. smegmatis* mc^2155 containing the parental pMV261 plasmid was used as control to examine the growth and CFU patterns. Among the various stressful conditions in this study, the results showed that the stressful conditions as exposure with reactive oxygen intermediate and cultivation under nutritional starvation exhibited the significant difference

(p<0.05) of growth or survival rates from control and test strains. After exposure with reactive oxygen intermediate, the survival rate of *M. smegmatis* mc^2155 containing *relE* gene was rapidly decreased with the increasing concentrations of hydrogen peroxide as 70%, 10% and 1% of survival rates with concentrations at 5, 10 and 20 mM of hydrogen peroxide, respectively. Whereas, the growth rate of control strain was slightly decrease as 85%, 70% and 56% of survival rates with concentrations at 5, 10 and 20 mM of hydrogen peroxide, respectively. Similarity to the previous stressful condition, CFU of test cells was rapidly decreased until it was completely dead at 6^{th} and 7^{th} day during cultivation in completely starved culture. In contrast, CFU of control cells from deionized water was remained constant at 10^4 CFU/ml at all time points after it was slightly dropped in an initial period. No significant difference ($p<0.05$) between test and control strains was found under acid condition, exposure with reactive nitrogen intermediate and high temperature.

BIBLIOGRAPHY

Agarwal R., Malhotra P., Awasthi A., Kakkar N. and Gupta D. 2005. "Tuberculous dilated cardiomyopathy: an under-recognized entity." **BMC Infect Dis.** 5(1): 29.

Aizenman E., Engelberg-Kulka H. and Glaser G. 1996. "An *Escherichia coli* chromosomal addiction module regulated by guanosine-3959-bispyrophosphate: A model for programmed bacterial cell death." **Proc. Natl. Acad. Sci. USA.** 93: 6059-6063.

Akaki T., Sato K., Shimizu T., Sano C., Kajitani H., Dekio S. and Tomioka H. 1997. "Effector molecules in expression of the antimicrobial activity of macrophages against *M. avium* complex: roles of reactive nitrogen intermediates, reactive oxygen intermediates, and free fatty acids." **J. Leukoc. Biol.** 62: 795-804.

Anantharaman V. and Aravind L. 2003. "New connections in the prokaryotic toxin–antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system." **Genome Biol.** 4: 81.

Barry C.E., Lee R.E., Mdluli K., Sampson A.E., Schroeder B.G., Slayden R.A. and Yuan Y. 1998. "Mycolic acids: structure, biosynthesis and physiological functions." **Progr. Lid. Res.** 37: 143-179.

Bech F.W., Jorgensen S.T., Diderichsen B. and Karlstrom O.H. 1985. "Sequence of the *relB* transcription unit from *Escherichia coli*." **EMBO. J.** 4: 1059-1066.

Betts J.C., Lukey P.T., Robb L.C., McAdam R.A. and Duncan K. 2002. "Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling." **Mol. Microbiol.** 43: 717-731.

Bravo A., de Torrontegui G. and Diaz R. 1987. "Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid." **Mol. Gen. Genet.** 210: 101-110.

Brookes H.R., Hill C.P., Owiafe K.P., Ibanga B.H., Jeffries J.D., Donkor A.S., Fletcher A.H., Hammond S.A., Lienhardt C., Adegbola A.R., McShane H. and Adrian V.S. 2008. "Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa.". **PLOS ONE.** 3(8): 2921.

Brown M.J. and Shaw J. K. 2003. "A novel family of *Escherichia coli* toxin-antitoxin gene pairs." **J. Bacterioal.** 185(22): 6600-6608.

Camus J.C., Pryor M.J., Medigue C. and Cole S.T. 2002. "Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv." **Microbiology J.** 148: 2967-2973.

Cardona P.J. and Ruiz-Manzano J. 2004. "On the nature of *Mycobacterium tuberculosis*-latent bacilli." **Eur. Respir. J.** 24: 1044-1051.

CDC (The centers for disease control and prevention). 2006. "Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs-worldwide, 2000-2004 (2006)." **MMWR. Morb. Mortal Wkly. Rep.** 55(11): 301-305.

Choi K.H., Laurent Kremer L., Besra S.G. and Rock O.C. 2000. "Identification and Substrate Specificity of β-Ketoacyl (Acyl Carrier Protein) Synthase III (mtFabH) from *Mycobacterium tuberculosis*." **J. Biol. Chem.** 275 (36): 28201-28207.

Chopra P., Meena S.L. and Singh Y. 2003. "New drug targets for *Mycobacterium tuberculosis*." **Indian J. Med. Res.** 117: 1-9.

Christensen S.K., Mikkelsen M., Pedersen K. and Gerdes K. 2001. "RelE, a global inhibitor of translation, is activated during nutritional stress." **Proc. Natl. Acad. Sci. USA.** 98:14328-14333.

Christensen S.K. and Gerdes K. 2003. "RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA." **Mol. Microbiol.** 48: 1389-1400.

Christensen K.S. and Gerdes K. 2004. "Delayed-relaxed response explained by hyperactivation of RelE." **Mol. Microbiol.** 53(2): 587-597

Colangeli R., Haq A., Arcus V.L., Summersb E., Magliozzoc S.R., McBridea A., Mitrad K.A., Radjainiad M., Khajoc A., Jacobs R.W., Salgamea P. and Allanda D. 2008. "The multifunctional histone-like protein Lsr2 protects mycobacteria against reactive oxygen intermediates." **PNAS.** 106(11): 4414-4418.

Cole S.T., Brosch R., Parkhill J., Garnier T., Churcher C., Harris D., Gordon S.V., Eiglmeier K., Gas S., Barry C.E., Tekaia F., Badcock K., Basham D., Brown D., Chillingworth T., Connor R., Davies R., Devlin K., Feltwell T., Gentles S., Hamlin N., Holroyd S., Hornsby T., Jagels K., Krogh A., McLean J., Moule S., Murphy L., Oliver K., Osborne J., Quail M.A., Rajandream M.A., Rogers J., Rutter S., Seeger K., Skelton J., Squares R., Squares S., Sulston J.E., Taylor K., Whitehead S., Barrell B.G. 1998. "Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence." **Nature.** 393: 537-544.

Condon C. 2006. "Shutdown decay of mRNA." **Mol. Microbiol.** 61(3): 573-583.

Couturier M., Bahassi E.M. and Van Melderen L. 1998. "Bacterial death by DNA gyrase poisoning." **Trends Microbiol.** 6: 269-275.

Dermine F.J. and Desjardins M. 1999. "Survival of intracellular pathogens within macrophages." **Protoplasma.** 210: 1615-6102.

Diderichsen B., Fiil N.P. and Lavalle R. 1977. "Genetics of the *relB* locus in *Escherichia coli.*" **J. Bacteriol.** 131: 30-33.

Edwards D. and Kirkpatrick C.H. 1986. "The immunology of mycobacterial diseases." **Am. Rev. Respir. Dis.** 134:1062–1071.

Engelberg K., Hazan R. and Amitai S. 2005. "*mazEF*: a chromosomal toxin–antitoxin module that triggers programmed cell death in bacteria." **J. Cell Science.** 118: 4327-4332.

Ernst JD., Trevejo-Nuñez G. and Banaiee N. 2007. "Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis." **J. Clin. Invest.** 117(7): 1738-1745.

Faridani R.O., Nikravesh A., Pandey P.O., Gerdes K. and Good L. 2006. "Competitive inhibition of natural antisense Sok-RNA interactions activates Hok-mediated cell killing in *Escherichia coli.*" **J. Nucl. Acid Res.** (34)20: 5915-5922.

Fine P., Floyd S., Stanford J., Nkhosa P., Kasunga A., Chaguluka S., Warndorff D., Jenkins P., Yates M. and Ponnighaus J. 2001. "Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy." **Epidemiol. Infect.** 126(3): 379-387.

Franch T. and Gerdes K. 1996. "Programmed cell death in bacteria: translational repression by mRNA end-pairing." **Mol. Microbiol.** 21: 1049-1060.

Franch T., Gultyaev P.A. and Gerdes K. 1997. "Program cell death by *hok/sok* of plasmid R1 : Processing at the *hok* mRNA 3' end triggers structural rearrangements that allow translation and antisense RNA binding." **J. Mol. Biol.** 273: 38-51.

Furuya N. and Komano T. 1996. "Nucleotide sequence and characterization of the *trbABC* region of the IncI1 Plasmid R64: existence of the *pnd* gene for plasmid maintenance within the transfer region." **J. Bacteriol.** 178: 1491-1497.

Galvani C., Terry J. and Ishiguro E.E. 2001. "Purification of the RelB and RelE Proteins of *Escherichia coli*: RelE binds RelB and to ribosomes." **J. Bacteriol.** 183: 2700-2703.

Gerdes K., Rasmussen P.B. and Molin S. 1986. "Unique type of plasmid maintenance function: postsegregational killing of plasmid free cells." **Proc. Natl. Acad. Sci.** 83: 3116-3120.

Gerdes K., Poulsen L.K., Thisted T., Nielsen A.K., Martinussen J. and Andreasen P.H. 1990. "The *hok* killer gene family in gram-negative bacteria." **New Biol.** 2: 946-956.

Gerdes K., Nielsen A., Thorsted P. and Wagner E.G. 1992. "Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable *hok, srnB* and *pndA* effector messenger RNAs." **J. Mol. Biol.** 226: 637-649.

Gerdes K., Gultyaev A.P., Franch T., Pedersen K. and Mikkelsen N.D. 1997. "Antisense RNA regulated programmed cell death." **Annu. Rev. Genet.** 31: 1-31.

Gerdes K. 2000. "Toxin-Antitoxin modules may regulate synthesis of macromolecules during nutritional stress." **J. bacterioal.** 182(3): 561-572.

Gerdes K., Christensen S.K. and Lobner-Olesen A. 2005. "Prokaryotic toxin-antitoxin stress response loci." **Nature Rev. Microbiol.** 3: 371-382.

Gillespie H.S. 2002. " Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective." **Antimicrob. Agents Chemother.** 46(2): 267-274.

Glickman J. 1994. "Microbial Pathogenesis of *Mycobacterium tuberculosis*: Dawn of a Discipline." **Cell.** 104(4): 477-485.

Gotfredsen M. and Gerdes K. 1998. "The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family." **Mol. Microbiol.** 29: 1065–1076.

Grady R. and Hayes F. 2003. "Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrugresistant, clinical isolate of *Enterococcus faecium*." **Mol. Microbiol.** 47: 1419-1432.

Gronlund H. and Gerdes K. 1999. "Toxin–antitoxin systems homologous with *relBE* of *Escherichia coli* plasmid P307 are ubiquitous in prokaryotes." **J. Mol. Biol.** 285: 1401-1415.

Gupta A. 2008. "Killing activityand rescue function of genome-wide toxin-antitoxin loci of *Mycobacterium tuberculosis*." **FEMS Microbiol Lett.** 290: 45–53.

Hazan R. and Engelberg-Kulka H. 2004. "*Escherichia coli mazEF*-mediated cell death as a defense mechanism that inhibits the spread of phage P1." **Mol. Genet. Genomics.** 272: 227-234.

Hazan R., Sat B. and Engelberg-Kulka H. 2004. "*Escherichia coli mazEF*-mediated cell death is triggered by various stressful conditions." **J. Bacteriol.** 186:3663-3669.

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

Houben E., Nguyen L. and Pieters J. 2006. "Interaction of pathogenic mycobacteria with the host immune system". **Curr. Opin. Microbiol.** 9(1): 76-85.

Iivanainen E., Martikainen P.J., Vaananen P. and Katila M. L. 1999. "Environmental factors affecting the occurrence of mycobacteria in brook sediments. **J. Appl. Microbiol.** 86: 673-681.

Jensen R.B. and Gerdes K. 1995. "Programmed cell death in bacteria: proteic plasmid stabilization systems." **Mol. Microbiol.** 17: 205-210.

John C., Yu T., Kathryn E.T., Ming S.T., Keming Y., Padmini S., Dinah C., Yvonne K., Rachel T. and Barry R.B. 1996. "Effects of protein calorie malnutrition on tuberculosis in mice." **Proc. Natl. Acad. Sci. USA.** 93(25): 14857-14861.

Kamada K., Hanaoka F. and Burley S.K. 2003. "Crystal structure of the MazE/MazF complex: molecular bases of antidote-toxin recognition." **Mol. Cell.** 11: 875-884.

Karzai A.W., Roch E.D. and Sauer R.T. 2000. "The SsrA-Smp system for protein tagging, directed degradation and ribosome rescue." **Nat. Struct. Biol.** 7: 449-455.

Keiler K.C., Waller P.R. and Sauer R.T. 1996. "Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA." **Science.** 271: 990-993.

Kim J., Park Y., Kim Y., Kang S., Shin J., Park I. and Choi B. 2003. "Miliary tuberculosis and acute respiratory distress syndrome." **Int. J. Tuberc. Lung Dis.** 7(4): 359-364.

Konomi N., Lebwohl E., Mowbray K., Tattersall I. and Zhang D. 2002. "Detection of mycobacterial DNA in Andean mummies." **J. Clin. Microbiol.** 40(12): 4738-4740.

Korch B.S., Contreras H. and Clark-Curtiss E.J. 2009. "Three Mycobacterium tuberculosis Rel Toxin-Antitoxin Modules Inhibit Mycobacterial Growth and Are Expressed in Infected Human Macrophages." **J. Bacteriol.** 191(5): 1618–1630.

Kuroda A., Nomura K., Ohtomo R., Kato J., Ikeda T. and Takiguchi N. 2001. "Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli.*" **Science.** 293: 705-708.

Lehnherr H., Maguin E., Jafri S. and Yarmolinsky M.B. 1993. "Plasmid addiction genes of bacteriophage P1: doc, which causes cell death on curing of prophage, and *phd*, which prevents host death when prophage is retained." **J. Mol. Biol.** 233: 414-428.

Lewis K. 2000. "Programmed death in bacteria." **Mol. Biol. Rev.** 64:503–514.

Li G.Y., Zhang Y., Chan M.C., Mal T.K., Hoeflich K.P., Inouye M. and Ikura M. 2006. "Characterization of dual substrate binding sites in the homodimeric structure of *Escherichia coli* mRNA interferase MazF." **J. Mol. Biol.** 357: 139-150.

Li G.Y., Zhang Y., Inouye M. and Ikura1 M. 2008. "Structural Mechanism of Transcriptional Autorepression of the *Escherichia coli* RelB/RelE Antitoxin/Toxin." **J. Mol. Biol.** 380(1):107-119.

Lindquist S. 1986. "Overexpression of heat-shock proteins reduces survival of Mycobacterium tuberculosis in the chronic phase of infection." **Annu. Rev. Biochem.** 55:1151-1191.

Loh S.M., Cram D.S. and Skurray, R.A. 1988. "Nucleotide sequence and transcriptional analysis of a third function (*Flm*) involved in F-plasmid maintenance." **Gene.** 66: 259-268.

Marra F., Marra A.C., Bruchet N., Richardson K., Moadebi S., Elwood K.R. and FitzGerald M.J. 2007. "Adverse drug reactions associated with first-line anti-tuberculosis drug regimens." **Int. J. Tuberc. Lung Dis.** 11(8):868-875.

Martin C. 2006. "Tuberculosis vaccines: past, present and future." **Curr. Opin. Pulm. Med.** 12 (3): 186-191.

Masuda Y., Miyakawa K., Nishimura Y. and Ohtsubo E. 1993. "*chpA* and *chpB*, *Escherichia coli* chromosomal homologs of the *pem* locus responsible for stable maintenance of plasmid R100." **J. Bacteriol.** 175 : 6850-6856.

Metzger S., Dror I.B., Aizenman E., Schreiber G., Toone M. and Friesen J.D. 1988. "The nucleotide sequence and characterization of the *relA* gene of *Escherichia coli*." **J. Biol. Chem.** 263: 15699-15704.

Minnikin DE. 1982. "Lipids: complex lipids, their chemistry, biosynthesis and roles." **The biology of the mycobacteria.** 1: 95-184.

Mitnick C., Bayona J., Palacios E., Sonya Shin R.N., Furin J., Alcántara F., Sánchez E., Sarria M., Becerra M., Fawzi S.C., Kapiga S., Neuberg D., Maguire H.J., Kim Y.J. and Farmer P. 2003. "Community-based therapy for multidrug-resistant tuberculosis in Lima, Peru". **N. Eng. J. Med.** 348(2): 119-128.

Moller J.J., Franch T. and Gerdes K. 2001. "Temporal translation control by Metastable RNA structure" **J. Biol. Chem.** 276(38): 35707-35713.

Murray P. R., Rosenthal K. S. and Pfaller M. A. 2005. "Bacteriology: Mycobacterium." **Medical Microbiology.** Elsevier Mosby. 5: 234-248.

Muto A., Ushida C. and Himeno H. 1998. "A bacterial RNA that functions both as a tRNA and an mRNA." **Tanpakushitsu Kakusan Koso.** 43: 1433-1442.

Nahid P., Pai M. and Hopewell P. 2006. "Advances in the diagnosis and treatment of tuberculosis." **Proc. Am. Thorac. Soc.** 3(1): 103-110.

National Institute of Allergy and Infectious Diseases (NIAID). 2006. "According to the World Health Organization (WHO), nearly 2 billion people, one-third of the world's population, have TB."

Niemann S., Rusch-Gerdes S., Joloba L.M., Whalen C.C., Guwatudde D., Ellner J.J., Eisenach K., Fumokong N., Johnson J.L., Aisu T., Mugerwa R.D., Okwera A., and Schwander S.K. 2002. "*Mycobacterium africanum* subtype II is associated with two distinct genotypes and is a major cause of human tuberculosis in Kampala, Uganda." **J. Clin. Microbiol.** 40(9): 3398-3405.

Nishiyama Y., Massey V., Takeda K., Kawasaki S., Sato J., Watanabe T. and Niimura Y. 2001. "Hydrogen Peroxide-Forming NADH Oxidase Belonging to the Peroxiredoxin Oxidoreductase Family: Existence and Physiological Role in Bacteria." **J. Bacteriol.** 183(8): 2431-2438.

Noss H.E., Pai K.R., Sellati J.T., Radolf D.J., Belisle J., Golenbock T.D., Boom H.W. and Harding V.C. 2001. "Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*." **J. Immunol.** 167: 910-918.

Nozaki Y., Hasegawa Y., Ama S., Nakashima I. and Shimokata K. 1997. "Mechanism of Nitric Oxide-Dependent Killing of Mycobacterium bovis BCG in Human Alveolar Macrophages." Infect. Immun. 65(9): 3644–3647.

Nyka W. 1974. "Studies on the effects of starvation on mycobacteria." **Infect. Immun.** 9:843–850.

Nyström T. 1994. "The glucose-starvation stimulon of *Escherichia coli*: induced and repressed synthesis of enzymes of central metabolic pathways and role of acetyl phosphate in gene expression and starvation survival." **Mol. Microbiol.** 12: 833-843.

Ogura T. and Hiraga S. 1983. "Mini-F plasmid genes that couple host cell division to plasmid proliferation." **Proc. Natl. Acad. Sci.** 80: 4784-4788.

Oh Y.K. and Straubinger R.M. 1996. "Intracellular fate of *Mycobacterium avium*: use of dual-label spectro¯uorometry to investigate the in¯uence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction." **Infect Immun** 64: 319-325.

Ono K., Akimoto S., Ono T. and Ohnishi, Y. 1986. "Plasmid genes increase membrane permeability in *Escherichia coli*." **Biochim. Biophys. Acta.** 867: 81-88.

Ono K., Akimoto S. and Ohnishi Y. 1987. "Nucleotide sequence of the *pnd* gene in plasmid R483 and role of the *pnd* gene product in plasmolysis." **Microbiol. Immunol.** 31: 1071-1083.

Onyebujoh P.R. and Graham A.W. 2006. "World Health Organization Disease Watch: Focus: Tuberculosis."

Ottenhoff T.H., Verreck F.A., Hoeve M.A. and van de Vosse E. 2005. "Control of human host immunity to mycobacteria." **Tuberculosis (Edinb).** 85(1-2):53-64.

Pai M., Zwerling A. and Menzies D. 2008. "Systematic Review: T-Cell-based assays for the diagnosis of latent tuberculosis infection: An Update." **Ann. Intern. Med.** 149(3): 1-9.

Pandey D.P. and Gerdes K. 2005. "Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes." **Nucl. Acids. Res.** 33: 966-976.

Pandey P.D. and Gerdes K. 2008. "Toxin–antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes." **J. Mol. Biol.** 380: 107-119.

Parrish N., Dick J. and Bishai W. 1998. "Mechanisms of latency in *Mycobacterium tuberculosis*". **Trends Microbiol.** 6(3): 107-112.

Pearce-Duvet J. 2006. "The origin of human pathogens: evaluating the role of agriculture and domestic animals in the evolution of human disease." **Biol. Rev. Camb. Philos. Soc.** 81(3): 369-382.

Pedersen K. and Gerdes K. 1999. "Multiple hok genes on the chromosome of *Escherichia coli*. **Mol. Microbiol.** 32: 1090-1102.

Pedersen K., Zavialov A.V., Pavlov M.Y., Elf J., Gerdes K. and Ehrenberg M. 2003. "The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site." **Cell.** 112: 131-140.

Piddington D.L., Kashkouli A. and Buchmeier N.A. 2000. Growth of *Mycobacterium tuberculosis* in a defined medium is very restricted by acid pH and Mg^{+} levels. **Infect. Immun.** 68:4518-4522.

Pliny E., Ramachandra L., Noss E., Boom H.W. and Harding V.C. 2001. "Processing of *Mycobacterium tuberculosis* antigen 85B involves intraphagosomal formation of peptide–major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation." **J. Exp. Med.** 194(10): 1421-1432.

Poulsen L.K., Larsen N.W., Molin S. and Andersson P. 1989. "A family of genes encoding a cell-killing function may be conserved in all gram-negative bacteria." **Mol. Microbiol.** 3: 1463-1472.

Portaels F. and Pattyn S.R. 1982. "Growth of mycobacteria in relation to the pH of the medium." **Ann. Inst. Pasteur.** 133: 213-221.

Primm P.T., Andersen J.S., Mizrahi V., Avarbock D., Rubin H. and Barry E.C. 2000. "The Stringent Response of *Mycobacterium tuberculosis* Is Required for Long-Term Survival." **J Bacteriol.** 182(17): 4889–4898.

Pullinger G.D. and Lax A.J. 1992. "A Salmonella dublin virulence plasmid locus that affects bacterial growth under nutrient-limited conditions." **Mol. Microbiol.** 6: 1631-1643.

Rao M., Streur L.T., Aldwell E.F. and Cook M.G. 2001. "Intracellular pH regulation by *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG." **Microbiology**. 147: 1017–1024.

Rastogi N. 1991. "Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration or a model in terms of mycobacterial pathogenicity, virulence and drug resistance." **Res. Micrabiol.** 142: 464-476.

Raviglione M.C. and Luelmo F.L. 1996. "Update on the global epidermiology of tuberculosis." **Current Issues Public Health.** 2: 192-197.

Rieder H.L. 1999. "Epidemiologic basis of tuberculosis control." **International Union Against Tuberculosis and Lung Disease.** 1: 123-131.

Roberts R.C. and Helinski D.R. 1992. "Definition of a minimal plasmid stabilization system from the broad-host-range plasmid RK2." **J. Bacteriol.** 174: 8119-8132.

Rothel J. and Andersen P. 2005. "Diagnosis of latent Mycobacterium tuberculosis infection: is the demise of the Mantoux test imminent." **Expert Rev. Anti. Infect. Ther.** 3(6): 981-93.

Sakikawa T., Akimoto S. and Ohnishi Y. 1989. "The *pnd* gene in *E. coli* plasmid R16: Nucleotide sequence and gene expression leading to cell Mg^{2+} release and stable RNA degradation." **Biochim. Biophys. Acta.** 1007: 158-166.

Sat B., Hazan R., Fisher T., Khaner H., Glaser G. and Engelberg-Kulka H. 2001. "Programmed cell death in *Escherichia coli*: some antibiotics can trigger *mazEF* lethality." **J. Bacteriol.** 183: 2041-2045.

Sat B., Reches M. and Engelberg-Kulka H. 2003. "The *Escherichia coli mazEF* suicide module mediates thymineless death." **J. Bacteriol.** 185: 1803-1807.

Schluger W.N. and Rom N.W. 1998. "The Host Immune Response to Tuberculosis." **Am. J. Respir. Crit. Care Med.** 157: 679-691.

Sevin E.W. and Barloy-Hubler F. 2007. "RASTABacteria: a web-based tool for identifying toxin–antitoxin loci in prokaryotes." **Genome Biol.** 8: 155.

Singh J.A., Upshur R. and Padayatchi N. 2007. "XDR-TB in South Africa: No Time for Denial or Complacency.". **PloS Med.** 4(1): 50.

Snapper B.A., Melton E.R., Mustafa S., Kieser T. and Jacobs Jr R.W. 1990. "Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium Smegmatis*," **Mol. Microbiol.** 4: 1911-1919.

St. John G., Brot N., Ruan J., Erdjument-Bromage H., Tempst P., Weissbach H. and Nathan C. 2001. "Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates." **Proc Natl Acad Sci U S A.** 98(17): 9901–9906.

Stephen K.F. and Robert L.C. 2006. "Lung disease due to the more common nontuberculous mycobacteria." **Clin. Chest. Med.** 129: 1653-1672.

Stewart G.R., Snewin A.V., Walzl G., Hussell T., Tormay P., Gaora O.P., Goyal M., Betts J., Brown N.I. and Young B.D. 2001. "Overexpression of heat-shock proteins reduces survival of *Mycobacterium tuberculosis* in the chronic phase of infection." **Nature Medicine.** 7: 732 – 737.

Stewart G.R., Wernisch L., Stabler R., Mangan J.A., Hinds J., Laing K.G., Young B.D. and Butcher D.P. 2002. "Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays." **J.Microbiology.** 148:3129-3138.

Stover C.K., de la Cruz V.F., Fuerst T.R., Burlein J.E., Benson L.A., Bennett L.T., Bansal G.P., Young J.F., Lee M.H. and Hatfull G.F. 1991. "New use of BCG for recombinant vaccines." **Nature.** 351: 456-460.

Sturgill-Koszycki S., Schlesinger P.H. and Chakraborty P. 1994. "Lack of acidication in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase." **Science.** 263: 678-681.

Strachan D.P., Powell K.J., Thaker A., Millard F.J. and Maxwell J.D. 1995. "Vegetarian diet as a risk factor for tuberculosis in immigrant south London Asians". **Thorax.** 50(2): 175-180.

Susanne K., Christensen K.S., Mikkelsen M., Pedersen K. and Gerdes K. 2001. "RelE, a global inhibitor of translation, is activated during nutritional stress." **Proc. Natl. Acad. Sci. USA.** 98: 14328-14333.

Takagi H., Kakuta Y., Okada T., Yao M., Tanaka I., and Kimura M. 2005. "Crystal structure of archaeal toxin antitoxin RelE-RelB complex with implications for toxin activity and antitoxin effects." **Nat. Struct. Mol. Biol.** 12: 327-331.

Teixeira H.C., Abramo C. and Munk M.E. 2007. "Immunological diagnosis of tuberculosis: problems and strategies for success." **J. Bras. Pneumol.** 33(3): 323-334.

Thisted T. and Gerdes K. 1992. "Mechanism of post-segregational killing by the *hok/sok* system of plasmid R1. Sok antisense RNA regulates *hok* gene expression indirectly through the overlapping *mok* gene." **J. Mol. Biol.** 223: 41-54.

Thisted T., Nielsen A.K. and Gerdes K. 1994a. "Mechanism of post-segregational killing: translation of Hok, SrnB and Pnd mRNAs of plasmids R1, F and R483 is activated by 30 end processing." **EMBO J.** 13: 1950-1959.

Thisted T., Sorensen N.S., Wagner E.G. and Gerdes K. 1994b. "Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 50 end single-stranded leader and competes with the 30 end of Hok mRNA for binding to the *mok* translational initiation region." **EMBO J.** 13: 1960-1968.

Thisted T., Sorensen N.S. and Gerdes K. 1995. "Mechanism of post-segregational killing: secondary structure analysis of the entire Hok mRNA from plasmid R1 suggests a fold-back structure that prevents translation and antisense RNA binding." **J. Mol. Biol.** 247: 859-873.

Tian Q.B., Ohnishi M., Tabuchi A. and Terawaki Y. 1996. "A new plasmid-encoded proteic killer gene system: cloning, sequencing, and analyzing *hig* locus of plasmid Rts1." **Biochem. Biophys. Res. Commun.** 220: 280-284.

Tu G.F., Reid G.E., Zhang J.G., Moritz R.L. and Simpson R.J. 1995. "C-terminal extension of truncated recombinant proteins in *Escherichia coli* with a 10Sa RNA decapeptide." **J. Biol. Chem.** 270: 9322-9326.

Voskuil M. I., Schnappinger D., Visconti K.C., Harrell M.I., Dolganov G.M., Sherman D.R., and Schoolnik G.K. 2003. "Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program." **J. Exp. Med.** 198:705–713.

Warner F.D. and Mizrahi V. 2006. "Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy." **Clin. Microbiol. Rev.** 19: 558-570.

Weaver K.E. and Clewell D.B. 1989. "Construction of *Enterococcus faecalis* pAD1 miniplasmids: Identification of a minimal pheromone response regulatory region and evaluation of a novel pheromone-dependent growth inhibition." **Plasmid.** 22: 106-119.

World Health Organization. 1992. "Tuberculosis notifi cation update." **WHO/ TB.** 169.

World Health Organization. 2006a. "Global tuberculosis control: surveillance, planning, financing." **WHO report 2006.** 362.

World Health Organization. 2006b. **Tuberculosis Fact sheet N°104 - Global and regional incidence.**

Zager M.E. and McNerney R. 2008. " Multidrug-resistant tuberculosis." **J. Infect. Dis.** 8(10): 1-5.

Zainuddin Z.F., Kunze Z.M. and Dale J.W. 1988. "Transformation of *Mycobacterium smegmatis* with *Escherichia coii* plasmids carrying a selectable resistance marker." **Mol. Micro.** 3(1): 29-34.

Zhang J. and Inouye M. 2002. "MazG, a nucleoside triphosphate pyrophosphohydrolase, interacts with Era, an essential GTPase in *Escherichia coli.*" **J. Bacteriol.** 184: 5323-5329.

Zhang Y., Scorpio A., Nikaido H. and Sun Z. 1999. "Role of acid pH and decent efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. **J. Bacteriol** 181: 2044-2049.

Zhang Y., Zhang J., Hara H., Kato I. and Inouye M. 2005. "Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase." **J. Biol. Chem.** 280: 3143-3150.

Zhao L. and Zhang J. 2008. "Biochemical characterization of chromosome toxin-antitoxin system in *Mycobacterium tuberculosis.*" **FEBS.** 582:710-714.

Zhu L., Zhang Y., Teh J-S, Zhang J., Connell N., Rubin H. and Inouye M. 2006. "Characterization of mRNA interferases from *Mycobacterium tuberculosis.*" **J. Biol. Chem.** 281: 18638-18648.

Zielenkiewicz U. and Ceglowski P. 2001. "Mechanism of plasmid stable maintenance with special focus o plasmid addiction system." **Acta Biochimica Polonica.** (48)4: 1003-1023.

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

Available: http://en.wikipedia.org/wiki/Image:TB_Culture.jpg

Available : http://www.cdc.gov/tb/xdrtb/overview.htm

Available : http://www.globalhealthreporting.org/countries/Thailand.asp

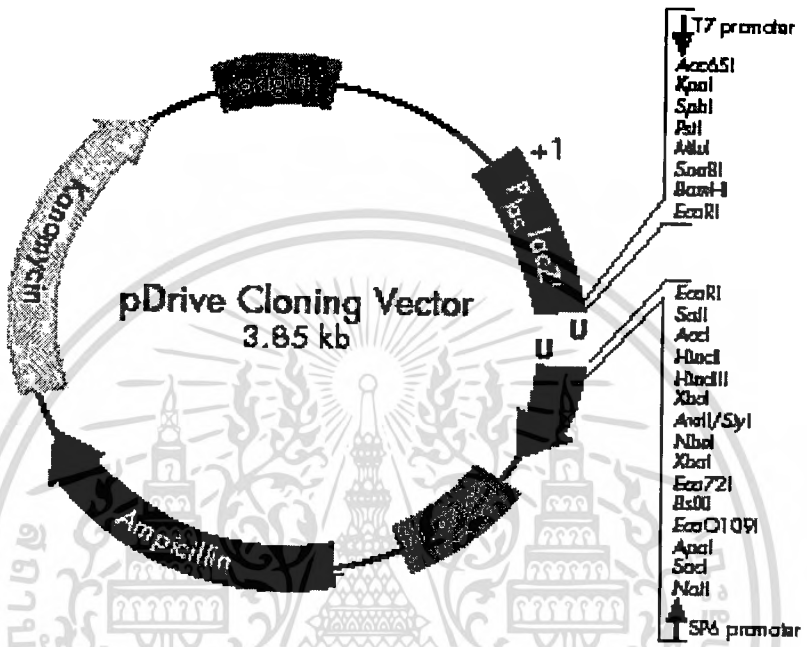
Available : http://www.in.gov/isdh/19686.htm

Available : http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=glyco2&part=ch20

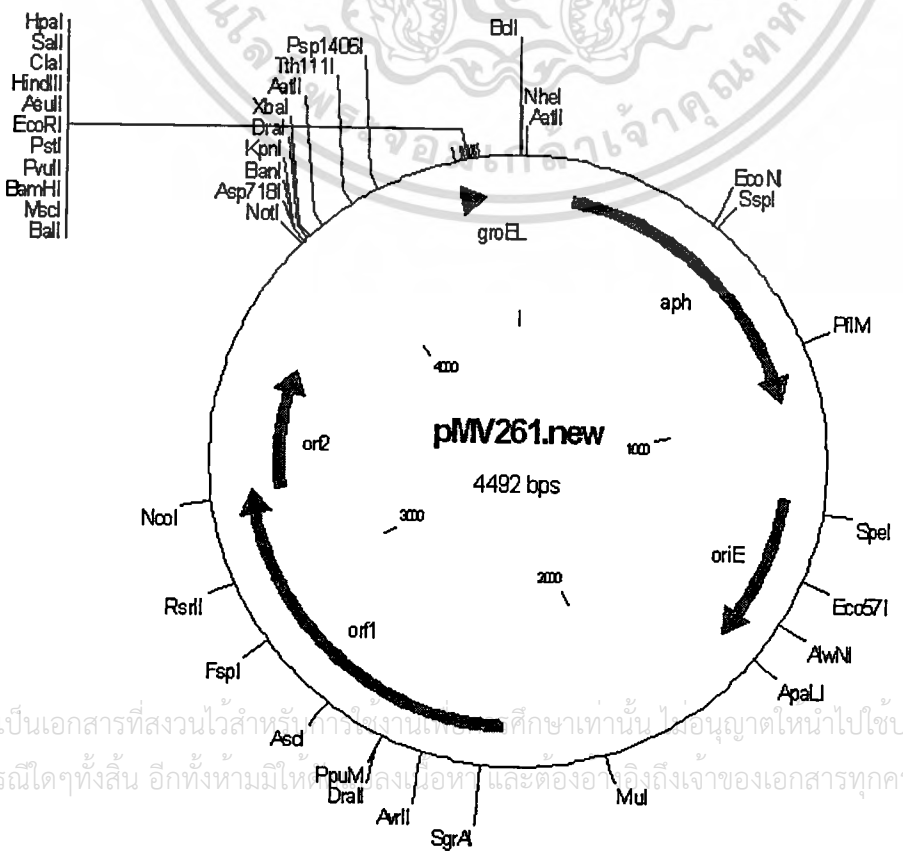
Available : http://www.tbdots.com/site/en/doctor_section_tb_diagnosis.html

APPENDIX A

1. Map of cloning vector pDrive (Qiagen, Hilden, Germany)



2. Map of *E. coli* / Mycobacterium shuttle vector pMV261



APPENDIX B

1. Luria-Bertani (LB) medium

LB broth

Ingredients (g/L)

Tryptone	10
Yeast extract	5
NaCl	10

LB agar

Ingredients (g/L)

Tryptone	10
Yeast extract	5
NaCl	10
Agar	15

pH 7.4

2. Middlebrook 7H10 medium

Ingredients (g/L)

Ammonium sulfate	0.50
L-Glutamic acid	0.50
Monopotassium phosphate	1.50
Disodium phosphate	1.50
Sodium citrate	0.40
Ferric ammonium citrate	0.04
Magnesium sulfate	0.025
Calcium chloride	0.0005
Zinc sulphate	0.001
Copper sulfate	0.001
Pyridoxine hydrochloride	0.001

APPENDIX B (CONTINUED)

Biotin	0.0005
Malachite green	0.00025
Agar	15.00
10% OADC	

3. SOB medium

Ingredients (g/L)

Tryptone	2.0
Yeast extract	5.0
Sodium chloride	0.5
Magnesium sulfate (anhydrous)	2.4
Potassium chloride	0.186

Table 1 Cell number of *M. smegmatis* mc^{2}155;:pMV261 and *M. smegmatis* mc^{2}155::pMV261-*relE* cultured in LB broth pH 7.4 containing 0.05% tween 80 and 50 μg/ml of kanamycin for 60 h.

Time of cultivation (hours)	Cell number (CFU/ml)	
	M. smegmatis mc^{2}155::pMV261	*M. smegmatis* mc^{2}155::pMV261-*relE*
0	$9.90\text{x}10^{4a} \pm 1.94$	$8.65\text{x}10^{4a} \pm 3.00$
6	$1.01\text{x}10^{5a} \pm 2.00$	$1.09\text{x}10^{5a} \pm 3.61$
12	$1.11\text{x}10^{5a} \pm 2.17$	$1.62\text{x}10^{5a} \pm 2.88$
24	$5.70\text{x}10^{5b} \pm 6.66$	$8.70\text{x}10^{5b} \pm 10.02$
30	$8.40\text{x}10^{6c} \pm 7.02\text{x}10$	$1.00\text{x}10^{7c} \pm 6.04\text{x}10$
36	$2.10\text{x}10^{8d} \pm 8.00\text{x}10^{2}$	$2.65\text{x}10^{8d} \pm 6.00\text{x}10^{2}$
48	$1.12\text{x}10^{9e} \pm 1.40\text{x}10^{4}$	$1.23\text{x}10^{9e} \pm 1.40\text{x}10^{4}$
54	$2.01\text{x}10^{9e} \pm 5.30\text{x}10^{4}$	$2.26\text{x}10^{9e} \pm 4.50\text{x}10^{4}$
60	$2.04\text{x}10^{9e} \pm 5.50\text{x}10^{4}$	$2.40\text{x}10^{9e} \pm 4.50\text{x}10^{4}$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 2 Cell number of *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) cultured in LB broth pH 5.5, 6.5 and 7.4 containing 0.05% Tween 80 and 50 µg/ml of kanamycin for 60 h.

Time (hours)	Cell number (CFU/ml)					
	pH5.5		pH 6.5		pH 7.4	
	Control	Test	Control	Test	Control	Test
0	$9.20\times10^{4a} \pm 2.45$	$8.74\times10^{4a} \pm 2.84$	$9.25\times10^{4a} \pm 3.21$	$8.57\times10^{4a} \pm 3.02$	$9.30\times10^{4a} \pm 1.94$	$8.65\times10^{4a} \pm 3.00$
6	$1.01\times10^{5a} \pm 3.42$	$9.92\times10^{4a} \pm 2.00$	$1.06\times10^{5a} \pm 3.47$	$9.25\times10^{4a} \pm 2.89$	$1.10\times10^{5a} \pm 2.00$	$9.90\times10^{4a} \pm 3.61$
12	$1.62\times10^{5a} \pm 4.18$	$1.57\times10^{5a} \pm 3.55$	$1.68\times10^{5a} \pm 3.41$	$1.51\times10^{5a} \pm 2.95$	$1.71\times10^{5a} \pm 2.17$	$1.52\times10^{5a} \pm 2.88$
24	$7.20\times10^{5b} \pm 7.31$	$6.70\times10^{5b} \pm 5.87$	$7.60\times10^{5b} \pm 9.60$	$6.50\times10^{5b} \pm 9.84$	$7.70\times10^{5b} \pm 6.66$	$6.70\times10^{5b} \pm 10.02$
30	$8.40\times10^{6c} \pm 8.07\times10$	$8.00\times10^{6c} \pm 8.65\times10$	$8.70\times10^{7c} \pm 7.23\times10$	$7.00\times10^{6c} \pm 6.93\times10$	$8.80\times10^{6c} \pm 7.02\times10$	$7.70\times10^{7c} \pm 6.04\times10$
36	$2.90\times10^{8d} \pm 7.34\times10^{2}$	$2.80\times10^{8d} \pm 8.25\times10^{2}$	$3.05\times10^{8d} \pm 6.83\times10^{2}$	$2.36\times10^{8d} \pm 6.42\times10^{2}$	$3.70\times10^{8d} \pm 8.00\times10^{2}$	$2.65\times10^{8d} \pm 6.00\times10^{2}$
48	$1.20\times10^{9e} \pm 1.21\times10^{4}$	$1.18\times10^{9e} \pm 1.29\times10^{4}$	$1.24\times10^{9e} \pm 1.33\times10^{4}$	$1.02\times10^{9e} \pm 1.53\times10^{4}$	$1.33\times10^{9e} \pm 1.40\times10^{4}$	$1.12\times10^{9e} \pm 1.40\times10^{4}$
54	$1.87\times10^{9e} \pm 5.42\times10^{4}$	$1.72\times10^{9e} \pm 5.72\times10^{4}$	$2.10\times10^{9e} \pm 4.86\times10^{4}$	$1.66\times10^{9e} \pm 4.78\times10^{4}$	$2.26\times10^{9e} \pm 4.50\times10^{4}$	$2.01\times10^{9e} \pm 5.30\times10^{4}$
60	$1.90\times10^{9e} \pm 5.70\times10^{4}$	$1.75\times10^{9e} \pm 5.83\times10^{4}$	$2.12\times10^{9e} \pm 5.50\times10^{4}$	$1.71\times10^{9e} \pm 5.01\times10^{4}$	$2.30\times10^{9e} \pm 4.50\times10^{4}$	$2.04\times10^{9e} \pm 5.50\times10^{5}$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

APPENDIX C (CONTINUED)

Table 3 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 and *M. smegmatis* mc^{2}155::pMV261-*relE* after exposure to either 0, 5, 10 and 20 mM of hydrogen peroxide for 2 h.

Concentration of H_2O_2 (mM)	Survival rate (%)	
	M. smegmatis mc^{2}155::pMV261	*M. smegmatis* mc^{2}155::pMV261-*relE*
0	$100^{a} \pm 15.15$	$100^{a} \pm 6.48$
5	$85.3^{a} \pm 13.24$	$66.92^{b} \pm 9.48$
10	$70.44^{a} \pm 5.15$	$11.36^{c} \pm 3.77$
20	$56.55^{b} \pm 4.77$	$1.30^{d} \pm 1.01$

The results are presented as (mean ± standard deviation) of five independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 4 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) after exposure to either 0, 5, 10, 15 and 20 mM of sodium nitrite for 0, 2, 4 and 6 h.

Concentration of $NaNO_2$ (mM)	Survival rate (%)							
	0 h		2 h		4 h		6 h	
	Control	Test	Control	Test	Control	Test	Control	Test
0	99.90^a ± 0.54	100.40^a ± 6.63	99.60^a ± 4.62	100.00^a ± 3.81	99.08^a ± 0.17	99.58^a ± 8.82	99.05^a ± 4.41	99.93^a ± 4.55
5	99.85^a ± 0.13	100.90^a ± 4.39	88.15^b ± 2.32	85.50^b ± 2.42	69.32^b ± 0.98	67.73^b ± 5.50	60.08^c ± 6.10	58.45^c ± 3.13
10	98.98^a ± 1.59	99.88^a ± 3.62	86.98^b ± 3.60	82.29^b ± 2.32	59.36^c ± 0.65	61.24^c ± 5.30	51.77^c ± 5.49	50.44^c ± 3.41
15	101.40^a ± 2.07	99.37^a ± 4.42	76.74^b ± 2.52	73.35^b ± 2.37	51.24^c ± 0.67	51.15^c ± 6.26	45.35^c ± 3.78	42.47^c ± 2.27
20	99.84^a ± 0.15	99.48^a ± 2.53	70.72^b ± 1.23	67.99^b ± 1.14	46.41^c ± 0.82	44.2^c ± 7.78	36.55^d ± 4.86	30.17^d ± 2.56

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 5 Cell number of *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) cultured in LB broth and deionized water.

Time of cultivation (days)	Cell number (CFU/ml)			
	LB broth		Deionized water	
	control	test	Control	Test
0	$1.20x10^{5a} \pm 4.16x10^{4}$	$1.20x10^{5a} \pm 1.53x10^{4}$	$1.53x10^{5a} \pm 3.79x10^{4}$	$1.50x10^{4a} \pm 1.00x10^{3}$
1	$5.60x10^{5a} \pm 5.86x10^{4}$	$5.50x10^{5a} \pm 1.53x10^{4}$	$1.23x10^{5a} \pm 2.52x10^{4}$	$7.00x10^{3a} \pm 1.00x10^{2}$
2	$6.55x10^{8b} \pm 4.76x10^{7}$	$6.39x10^{8b} \pm 4.78x10^{7}$	$1.00x10^{5a} \pm 3.61x10^{3}$	$3.67x10^{2a} \pm 3.79x10$
3	$4.13x10^{9c} \pm 1.53 \times 10^{8}$	$3.63x10^{9c} \pm 1.53x10^{8}$	$7.33x10^{4d} \pm 5.77x10^{3}$	$2.00x10^{e} \pm 7.23$
4	$4.60x10^{9c} \pm 2.00 \times 10^{8}$	$4.30x10^{9c} \pm 3.00x10^{8}$	$5.33x10^{4d} \pm 2.08x10^{3}$	$4.00^{f} \pm 2.08 \times 1.53$
5	$4.66x10^{9c} \pm 1.53x10^{8}$	$4.33x10^{9c} \pm 1.35x10^{8}$	$3.20x10^{4d} \pm 2.00x10^{3}$	$2.00^{f} \pm 2.00x1.68$
6	$4.70x10^{9c} \pm 4.58x10^{8}$	$4.57x10^{9c} \pm 1.15x10^{8}$	$2.67x10^{4d} \pm 1.52x10^{3}$	$0^{f} \pm 0$
7	$4.70x10^{9c} \pm 1.00x10^{8}$	$4.60x10^{9c} \pm 1.64x10^{8}$	$2.56x10^{4d} \pm 1.67x10^{3}$	$0^{f} \pm 0$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

APPENDIX C (CONTINUED)

Table 6 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) after exposure to 37, 42, 47 and 50°C for 0, 20, 40 and 60 min.

Temperature (°C)	Survival rate (%)							
	0 min		20 min		40 min		60 min	
	Control	Test	Control	Test	Control	Test	Control	Test
37	$99.80^{a} \pm 0.18$	$100.19^{a} \pm 0.22$	$99.20^{a} \pm 0.54$	$99.92^{a} \pm 0.26$	$99.20^{e} \pm 0.54$	$99.46^{a} \pm 0.48$	$98.59^{a} \pm 0.65$	$99.19^{a} \pm 0.34$
42	$100.09^{a} \pm 0.76$	$100.56^{a} \pm 0.66$	$91.11^{a} \pm 1.57$	$89.74^{a} \pm 0.66$	$71.52^{b} \pm 3.99$	$65.90^{b} \pm 3.98$	$49.68^{c} \pm 1.58$	$38.51^{c} \pm 0.21$
47	$100.51^{a} \pm 0.21$	$100.39^{a} \pm 1.27$	$71.36^{b} \pm 2.89$	$68.20^{b} \pm 1.27$	$46.88^{c} \pm 5.79$	$40.00^{c} \pm 1.69$	$27.09^{d} \pm 2.50$	$20.35^{d} \pm 2.09$
52	$99.61^{a} \pm 0.42$	$98.87^{a} \pm 1.38$	$45.19^{c} \pm 4.17$	$40.91^{c} \pm 1.38$	$19.19^{d} \pm 2.74$	$16.74^{d} \pm 0.42$	$3.07^{e} \pm 0.40$	$1.43^{e} \pm 0.38$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 1 Cell number of *M. smegmatis* mc^{2}155::pMV261 and *M. smegmatis* mc^{2}155::pMV261-*relE* cultured in LB broth pH 7.4 containing 0.05% tween 80 and 50 µg/ml of kanamycin for 60 h.

Time of cultivation (hours)	Cell number (CFU/ml)	
	M. smegmatis mc^{2}155::pMV261	*M. smegmatis* mc^{2}155::pMV261-*relE*
0	$9.90\text{x}10^{4a} \pm 1.94$	$8.65\text{x}10^{4a} \pm 3.00$
6	$1.01\text{x}10^{5a} \pm 2.00$	$1.09\text{x}10^{5a} \pm 3.61$
12	$1.11\text{x}10^{5a} \pm 2.17$	$1.62\text{x}10^{5a} \pm 2.88$
24	$5.70\text{x}10^{5b} \pm 6.66$	$8.70\text{x}10^{5b} \pm 10.02$
30	$8.40\text{x}10^{6c} \pm 7.02\text{x}10$	$1.00\text{x}10^{7c} \pm 6.04\text{x}10$
36	$2.10\text{x}10^{8d} \pm 8.00\text{x}10^{2}$	$2.65\text{x}10^{8d} \pm 6.00\text{x}10^{2}$
48	$1.12\text{x}10^{9e} \pm 1.40\text{x}10^{4}$	$1.23\text{x}10^{9e} \pm 1.40\text{x}10^{4}$
54	$2.01\text{x}10^{9e} \pm 5.30\text{x}10^{4}$	$2.26\text{x}10^{9e} \pm 4.50\text{x}10^{4}$
60	$2.04\text{x}10^{9e} \pm 5.50\text{x}10^{4}$	$2.40\text{x}10^{9e} \pm 4.50\text{x}10^{4}$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 2 Cell number of *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) cultured in LB broth pH 5.5, 6.5 and 7.4 containing 0.05% Tween 80 and 50 µg/ml of kanamycin for 60 h.

Time (hours)	Cell number (CFU/ml)					
	pH5.5		pH 6.5		pH 7.4	
	Control	Test	Control	Test	Control	Test
0	$9.20\times10^{4a} \pm 2.45$	$8.74\times10^{4a} \pm 2.84$	$9.25\times10^{4a} \pm 3.21$	$8.57\times10^{4a} \pm 3.02$	$9.30\times10^{4a} \pm 1.94$	$8.65\times10^{4a} \pm 3.00$
6	$1.01\times10^{5a} \pm 3.42$	$9.92\times10^{4a} \pm 2.00$	$1.06\times10^{5a} \pm 3.47$	$9.25\times10^{4a} \pm 2.89$	$1.10\times10^{5a} \pm 2.00$	$9.90\times10^{4a} \pm 3.61$
12	$1.62\times10^{5a} \pm 4.18$	$1.57\times10^{5a} \pm 3.55$	$1.68\times10^{5a} \pm 3.41$	$1.51\times10^{5a} \pm 2.95$	$1.71\times10^{5a} \pm 2.17$	$1.52\times10^{5a} \pm 2.88$
24	$7.20\times10^{5b} \pm 7.31$	$6.70\times10^{5b} \pm 5.87$	$7.60\times10^{5b} \pm 9.60$	$6.50\times10^{5b} \pm 9.84$	$7.70\times10^{5b} \pm 6.66$	$6.70\times10^{5b} \pm 10.02$
30	$8.40\times10^{6c} \pm 8.07\times10$	$8.00\times10^{6c} \pm 8.65\times10$	$8.70\times10^{7c} \pm 7.23\times10$	$7.00\times10^{6c} \pm 6.93\times10$	$8.80\times10^{6c} \pm 7.02\times10$	$7.70\times10^{7c} \pm 6.04\times10$
36	$2.90\times10^{8d} \pm 7.34\times10^{2}$	$2.80\times10^{8d} \pm 8.25\times10^{2}$	$3.05\times10^{8d} \pm 6.83\times10^{2}$	$2.36\times10^{8d} \pm 6.42\times10^{2}$	$3.70\times10^{8d} \pm 8.00\times10^{2}$	$2.65\times10^{8d} \pm 6.00\times10^{2}$
48	$1.20\times10^{9e} \pm 1.21\times10^{4}$	$1.18\times10^{9e} \pm 1.29\times10^{4}$	$1.24\times10^{9e} \pm 1.33\times10^{4}$	$1.02\times10^{9e} \pm 1.53\times10^{4}$	$1.33\times10^{9e} \pm 1.40\times10^{4}$	$1.12\times10^{9e} \pm 1.40\times10^{4}$
54	$1.87\times10^{9e} \pm 5.42\times10^{4}$	$1.72\times10^{9e} \pm 5.72\times10^{4}$	$2.10\times10^{9e} \pm 4.86\times10^{4}$	$1.66\times10^{9e} \pm 4.78\times10^{4}$	$2.26\times10^{9e} \pm 4.50\times10^{4}$	$2.01\times10^{9e} \pm 5.30\times10^{4}$
60	$1.90\times10^{9e} \pm 5.70\times10^{4}$	$1.75\times10^{9e} \pm 5.83\times10^{4}$	$2.12\times10^{9e} \pm 5.50\times10^{4}$	$1.71\times10^{9e} \pm 5.01\times10^{4}$	$2.30\times10^{9e} \pm 4.50\times10^{4}$	$2.04\times10^{9e} \pm 5.50\times10^{5}$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 3 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 and *M. smegmatis* mc^{2}155::pMV261-*relE* after exposure to either 0, 5, 10 and 20 mM of hydrogen peroxide for 2 h.

Concentration of H_2O_2 (mM)	Survival rate (%)	
	M. smegmatis mc^{2}155::pMV261	*M. smegmatis* mc^{2}155::pMV261-*relE*
0	$100^{a} \pm 15.15$	$100^{a} \pm 6.48$
5	$85.3^{a} \pm 13.24$	$66.92^{b} \pm 9.48$
10	$70.44^{a} \pm 5.15$	$11.36^{c} \pm 3.77$
20	$56.55^{b} \pm 4.77$	$1.30^{d} \pm 1.01$

The results are presented as (mean ± standard deviation) of five independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

APPENDIX C (CONTINUED)

Table 4 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) after exposure to either 0, 5, 10, 15 and 20 mM of sodium nitrite for 0, 2, 4 and 6 h.

Concentration of $NaNO_2$ (mM)	Survival rate (%)							
	0 h		2 h		4 h		6 h	
	Control	Test	Control	Test	Control	Test	Control	Test
0	99.90^a ± 0.54	100.40^a ± 6.63	99.60^a ± 4.62	100.00^a ± 3.81	99.08^a ± 0.17	99.58^a ± 8.82	99.05^a ± 4.41	99.93^a ± 4.55
5	99.85^a ± 0.13	100.90^a ± 4.39	88.15^b ± 2.32	85.50^b ± 2.42	69.32^b ± 0.98	67.73^b ± 5.50	60.08^c ± 6.10	58.45^c ± 3.13
10	98.98^a ± 1.59	99.88^a ± 3.62	86.98^b ± 3.60	82.29^b ± 2.32	59.36^c ± 0.65	61.24^c ± 5.30	51.77^c ± 5.49	50.44^c ± 3.41
15	101.40^a ± 2.07	99.37^a ± 4.42	76.74^b ± 2.52	73.35^b ± 2.37	51.24^c ± 0.67	51.15^c ± 6.26	45.35^c ± 3.78	42.47^c ± 2.27
20	99.84^a ± 0.15	99.48^a ± 2.53	70.72^b ± 1.23	67.99^b ± 1.14	46.41^c ± 0.82	44.2^c ± 7.78	36.55^d ± 4.86	30.17^d ± 2.56

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 5 Cell number of *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) cultured in LB broth and deionized water.

Time of cultivation (days)	Cell number (CFU/ml)			
	LB broth		Deionized water	
	control	test	Control	Test
0	$1.20\times10^{5a} \pm 4.16\times10^{4}$	$1.20\times10^{5a} \pm 1.53\times10^{4}$	$1.53\times10^{5a} \pm 3.79\times10^{4}$	$1.50\times10^{4a} \pm 1.00\times10^{3}$
1	$5.60\times10^{5a} \pm 5.86\times10^{4}$	$5.50\times10^{5a} \pm 1.53\times10^{4}$	$1.23\times10^{5a} \pm 2.52\times10^{4}$	$7.00\times10^{3a} \pm 1.00\times10^{2}$
2	$6.55\times10^{8b} \pm 4.76\times10^{7}$	$6.39\times10^{8b} \pm 4.78\times10^{7}$	$1.00\times10^{5a} \pm 3.61\times10^{3}$	$3.67\times10^{2a} \pm 3.79\times10$
3	$4.13\times10^{9c} \pm 1.53\times10^{8}$	$3.63\times10^{9c} \pm 1.53\times10^{8}$	$7.33\times10^{4d} \pm 5.77\times10^{3}$	$2.00\times10^{e} \pm 7.23$
4	$4.60\times10^{9c} \pm 2.00\times10^{8}$	$4.30\times10^{9c} \pm 3.00\times10^{8}$	$5.33\times10^{4d} \pm 2.08\times10^{3}$	$4.00^{f} \pm 2.08\times1.53$
5	$4.66\times10^{9c} \pm 1.53\times10^{8}$	$4.33\times10^{9c} \pm 1.35\times10^{8}$	$3.20\times10^{4d} \pm 2.00\times10^{3}$	$2.00^{f} \pm 2.00\times1.68$
6	$4.70\times10^{9c} \pm 4.58\times10^{8}$	$4.57\times10^{9c} \pm 1.15\times10^{8}$	$2.67\times10^{4d} \pm 1.52\times10^{3}$	$0^{f} \pm 0$
7	$4.70\times10^{9c} \pm 1.00\times10^{8}$	$4.60\times10^{9c} \pm 1.64\times10^{8}$	$2.56\times10^{4d} \pm 1.67\times10^{3}$	$0^{f} \pm 0$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

Table 6 The percentage of survival rate for *M. smegmatis* mc^{2}155::pMV261 (control) and *M. smegmatis* mc^{2}155::pMV261-*relE* (test) after exposure to 37, 42, 47 and 50°C for 0, 20, 40 and 60 min.

Temperature (°C)	Survival rate (%)							
	0 min		20 min		40 min		60 min	
	Control	Test	Control	Test	Control	Test	Control	Test
37	$99.80^{a} \pm 0.18$	$100.19^{a} \pm 0.22$	$99.20^{a} \pm 0.54$	$99.92^{a} \pm 0.26$	$99.20^{a} \pm 0.54$	$99.46^{a} \pm 0.48$	$98.59^{a} \pm 0.65$	$99.19^{a} \pm 0.34$
42	$100.09^{a} \pm 0.76$	$100.56^{a} \pm 0.66$	$91.11^{a} \pm 1.57$	$89.74^{a} \pm 0.66$	$71.52^{b} \pm 3.99$	$65.90^{b} \pm 3.98$	$49.68^{c} \pm 1.58$	$38.51^{c} \pm 0.21$
47	$100.51^{a} \pm 0.21$	$100.39^{a} \pm 1.27$	$71.36^{b} \pm 2.89$	$68.20^{b} \pm 1.27$	$46.88^{c} \pm 5.79$	$40.00^{c} \pm 1.69$	$27.09^{d} \pm 2.50$	$20.35^{d} \pm 2.09$
52	$99.61^{a} \pm 0.42$	$98.87^{a} \pm 1.38$	$45.19^{c} \pm 4.17$	$40.91^{c} \pm 1.38$	$19.19^{d} \pm 2.74$	$16.74^{d} \pm 0.42$	$3.07^{e} \pm 0.40$	$1.43^{e} \pm 0.38$

The results are presented as (mean ± standard deviation) of three independent experiments. Values followed by a different letter indicate significant differences ($p < 0.05$). Values with shared letter are not significantly different ($p \geq 0.05$).

AUTHOR'S BIOGRAPHY

Wichanee Bankeeree was born in Bangkok, Thailand, on October 11, 1983. From 1995 to 2000, she studied high school at Depsirin Romklao School in science and mathematic program. She attended the King Mongkut's Institute of Technology Ladkrabang (KMITL) in 2001, and graduated bachelor's degree in biotechnology in 2006. After that, she began her master education in biotechnology at the same institute and eventually graduated in 2009.