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JUDUL: INHIBITING TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEM IN MYCOBACTERIUM TUBERCULOSIS

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INHIBITING TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEM IN *MYCOBACTERIUM TUBERCULOSIS*

JENIFER ROLLAND L

THIS DISSERTATION IS SUBMITTED
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DECLARATION

I hereby certify that this masterpiece of mine is my very own product except for the statements, abridgements and summaries that each of them has been explained its source.

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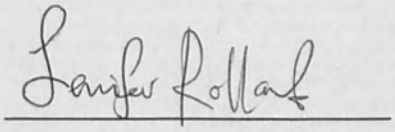

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ABSTRACT

In this work, my aim is to identify potential antimicrobial compounds that inhibit the regulation of two-component system in *Mycobacterium tuberculosis* over osmotic stress. Few diversity of actinomycetes were isolated from a total of 16 soil samples that had been collected from the virgin tropical rainforest of Danum Valley in Sabah. The soil samples were mainly collected under selected trees in the Newbery Plot area. A total of 46 strains of actinomycetes had been isolated from these soil samples through isolation using HV media and purification using Oatmeal media. Through fermentation process, different secondary metabolites were extracted from all these actinomycetes strains. The resulting acetone extracts were screened for K^+ and Na^+ signals in *Mycobacterium* two-component signal transduction system, on media that had been developed to apply osmotic pressure. *Mycobacterium smegmatis* was grown using the modified M9 minimal media with low and high concentration of potassium (K^+) and sodium (Na^+) ions. Wild-type *Mycobacterium smegmatis* mc^2155 strain H8000 was used as targeted microorganism in modified M9 minimal media. The pH of media was adjusted to 6.7. Glucose, thiamine hydrochloride (Vitamin B_1) and the inoculum of *M. smegmatis* were added into the media. The culture was incubated in 37°C for 3–4 days. Streptomycin was used as a negative control. A new simple but interesting discovery was found regarding streptomycin where its resulting inhibition zones were decreasing as the concentration of Na^+ signal in media arise. Besides reducing the growth of *M. smegmatis*, higher concentration of signals in the media had increased the media density, which might explain why the fixed concentration of streptomycin used as control cannot widen its inhibition zone. Only Extract H11588 showed antimicrobacterial activity on the two-component signal transduction system in *Mycobacterium.* However, it was later defined as toxic to *M. smegmatis* because it showed inhibition zones on both low and high concentrations (Na^+ concentration: Low: 0.8% (w/v); High: 4.0% (w/v); K^+ concentration: Low 100 μM; High: 1 mM) of both studied signals.

ABSTRAK

Objektif kajian ini adalah untuk mengenalpasti sebatian antimikrob yang berpotensi merencat pengawalaturan sistem dua-komponen di dalam *Mycobacterium tuberculosis* terhadap tekanan osmotik. Pelbagai aktinomiset telah diasingkan daripada sejumlah 16 sampel tanah yang diperolehi daripada hutan hujan tropika di Lembah Danum, Sabah. Sampel-sampel tanah diambil pada bahagian bawah pokok-pokok yang terpilih di dalam kawasan Plot Newbery. Sejumlah 46 strain aktinomiset telah berjaya dipencilkan daripada sampel-sampel tanah ini melalui kaedah pengasingan menggunakan media selektif HV dan kaedah penulenan menggunakan media kompleks Oatmeal. Melalui proses penapaian, pelbagai metabolit sekunder diekstrak daripada keseluruhan strain-strain aktinomiset ini. Ekstrak-ekstrak aseton yang terhasil diuji untuk isyarat K^+ dan Na^+ terhadap sistem isyarat transduksi dua-komponen *Mycobacterium* ke atas media yang telah diubahsuai untuk tekanan osmotik. *Mycobacterium smegmatis* dikulturkan pada media minimum M9 yang telah diubahsuai dengan kepekatan ion kalium (K^+) dan natrium (Na^+) yang tinggi dan rendah. *Mycobacterium smegmatis* mc^2155 jenis liar strain H8000 digunakan sebagai mikroorganisma sasaran. pH media diselaraskan kepada 6.7. Glukosa, thiamine hydrochloride (Vitamin B_1) dan inokulum *M. smegmatis* ditambahkan ke dalam media. Kultur kemudian dieramkan pada suhu 37°C selama 3–4 hari. Streptomycin digunakan sebagai kawalan negatif. Satu penemuan ringkas tetapi memberangsangkan mengenai streptomycin adalah di mana ia menghasilkan zon perencatan yang semakin kecil apabila kepekatan Na^+ semakin meningkat di dalam media. Kepekatan ion isyarat yang semakin tinggi dalam media telah meningkatkan kepadatan media, yang mana menjelaskan pengurangan zon perencatan yang dihasilkan oleh streptomycin. Peningkatan kepekatan K^+ dan Na^+ juga merencat pertumbuhan *M. smegmatis*, Hanya Ekstrak H11588 menunjukkan aktiviti antimikrobacteria terhadap sistem isyarat transduksi dua-komponen dalam *Mycobacterium*. Walau bagaimanapun, ia adalah toksik kepada *M. smegmatis* kerana menghasilkan zon perencatan pada kepekatan tinggi dan rendah (Kepekatan Na^+: Rendah: 0.8% (w/v); Tinggi: 4.0% (w/v); Kepekatan K^+: Rendah: 100 μM; Tinggi: 1 mM) untuk isyarat K^+ dan Na^+.

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SYMBOLS, UNITS, ABBREVIATIONS, TERMS & FORMULAS

µ	micro (10^{-6})
%	percentage
°C	degree Celsius
g	gram
mg	milligram
kg	kilogram
cm	centimeter
km	kilometer
m	meter
M	Molar
ml	milliliter
L	Liter
K^+	potassium ion
Na^+	sodium ion
No.	number
DV	Danum Valley
Std.	Standard
Mr.	Mister
Ms.	Miss
Prof.	Professor
HV	Humic acid – Vitamin B
r.p.m	Rotation per minute
ISO1	Isolation using Humic acid-Vitamin B agar media
PUR1	Purification using Oatmeal agar media
D	extract was diluted
UD	no dilution on extract
w/v	weight over volume
v/v	volume over volume

CHAPTER 1

INTRODUCTION

The need for novel approaches to control the regulation of tuberculosis is increasing. Approximately, 2–3 million people die because of this disease and it is believed that one-third of the world's population have been infected with *Mycobacterium tuberculosis*. The only widely used vaccine for tuberculosis is *Mycobacterium bovis* BCG, however, does not provide adequate levels of protection in Africa, India, and some parts of the USA (Rickman *et. al.*, 2004). Moreover, the increasing of drug-resistant strains of *M. tuberculosis* makes the identification of new drug targets and vaccines more challenging. One approach to combat tuberculosis is to identify new potential inhibitor via screening method that could inhibit a specific two-component system in *Mycobacterium tuberculosis*. Many bacteria, including *Mycobacterium*, use two-component signal transduction systems to allow them to respond rapidly to changes in their environment such as pH, temperature and osmotic stress, and control a variety of bacterial processes.

Potassium (K^+) and sodium (Na^+) were used to create the osmotic effects. Potassium is an essential macronutrient for the growth of most organisms. In bacteria, potassium plays an important role in the maintenance of intracellular pH and cell turgor. The second signal is sodium which also basically similar to potassium in terms of osmotic effect. The following are the steps in brief, which involved in screening. The bacteria were grown in the modified M9 minimal media (refer Table 3.3) with high and low concentrations of Na^+. Glucose (as the carbon source) and thiamine hydrochloride (Vitamin B_1) were added. Seed culture (liquid culture) of *Mycobacterium smegmatis* was prepared using the modified M9 minimal media and the bacteria were grown at 37°C temperature for 3–4 days. The screening step (refer Appendix B) subsequently took place in the same but solid (agar) media. Extracts were pipetted to different paper disc with 0.6 cm diameter. Streptomycin was used as the negative control. The media was comprised of two layers of agar- the upper layer (0.7 % w/v) and the bottom layer (1.0 % w/v). Glucose and thiamine hydrochloride (Vitamin B_1), as well as the seed culture of *Mycobacterium smegmatis* were added to upper layer only. The media were incubated at 37°C temperature for 4 days. These steps were repeated for studying K^+ signal (refer Appendix C) in modified M9 minimal media (refer Table 3.4) over osmotic stress.

For this work, the soil samples were collected from specific location of the Danum Valley Conservation Area. The Danum Valley Conservation Area is one of the last remaining preserves of primary lowland rainforest in Asia. Therefore, the search for novel potential inhibitors are greater as Danum Valley is one of Sabah's last strongholds of undisturbed lowland rain forest.

This work is purposely to identify a potential antimicrobial compound that inhibits the regulation of two-component system in *Mycobacterium tuberculosis* over osmotic stress. The scope is to grow *M. smegmatis* using the modified M9 minimal media and to identify potential inhibitor(s) in low concentration of potassium (K^+) and sodium (Na^{2+}) ions under optimum temperature of 37°C and almost neutral condition pH of 6.7. Other objectives of this work are (i) to collect a few soil samples from the virgin tropical rainforest of Danum Valley Conservation Area in Sabah, (ii) to isolate and to purify bacteria from the collected soil samples using respectively humic acid-Vitamin B agar and Oatmeal agar, (iii) to extract secondary metabolites from actinomycetes through fermentation process, (iv) to develop sufficient media for screening of actinomycetes for both signals, K^+ and Na^+, over osmotic pressure, (vi) to implement screening method of actinomycete for inhibitors of two-component system in *Mycobacterium smegmatis*, and finally, (vii) to practice screening method on extracts of actinomycetes.

CHAPTER 2

LITERATURE REVIEW

2.1 Actinomycetes

Actinomycetes are bacteria belonging to the order *Actinomycetales* and are characterized by the formation of branching filaments giving them a fungal appearance. Actinomycetes are widespread in nature and can be separated into two subgroups: oxidative forms, found mostly in soil habitants, and fermentative forms, living in natural cavities of man and animals. Actinomycete is a diverse and a large group of gram-positive filamentous and/or branching bacilli. These bacteria are in a form of Fungi-like bacteria that produce long, thread-like branched filaments that look like spider webs stretching through the compost. The earthy smell of compost is caused by actinomycetes. They are the primary decomposers of tough plant materials like bark, newspapers and woody stems. Actinomycetes are especially effective at attacking tough, raw plant tissues (cellulose, chitin and lignin), and softening them up for their less enterprising relatives. Most actinomycetes form spores, the manner of spores varies and is used in separating subgroups. The DNA base compositions of most members fall within the range of 68-

78% GC, and organisms at the upper end of this range have the highest GC percentage of any bacteria known (Madigan *et al.*, 2003).

Actinomycetes are saprophytes whose compost organic compounds in soil and act as parasite in hosts including human, which caused diseases like tuberculosis and leprosi. Species under this group are always in worldwide scientists' view because of their potential in producing antibiotics. For example, streptomycin from *Streptomyces griseus* for treatment of tuberculosis, which caused by *Mycobacterium tuberculosis*, and immunosuppressive drugs of takrolimus (FK506), which produced by *Streptomyces tsukubaensis* (Lo *et al.*, 2002). Actinomycetes are everywhere in nature, correct identification of *Nocardia* species is essential to rule out *Streptomyces* species that is usually considered nonpathogenic. Growth rate is from few days to 4 weeks. *Nocardia* species must also be compared with rapid grower mycobacteria that may mimic *Nocardia* both by macroscopically (culture) and microscopically (direct specimen or culture). Simple tests that are necessary to identify actinomycetes are growth rate, susceptibility to antibacterial agents, morphology information, distinct musty odor Gram stain, Modified Kinyoun Stain (MK), Ziehl-Neelsen Stain (ZN), Casein Hydrolysis and growth on selective and differential medium such as sodium pyruvate (PYR) for branching and fragmentation by *Nocardia* species. A recent study of two organisms (*Stachybotrys chartarum* and *Streptomyces califonicus)* commonly isolated from moisture-damaged structures indicated the organisms might increase their overall biomass when grown together in the laboratory as opposed to when each organism is grown individually. They are potentially hazardous individually and may also present another ser of problems when

growing together indoors, especially for prolonged periods of time. In contrast to the harmful effects, the actinomycetes also produce a majority of antibiotics that are beneficial to humans and animals (Rau, 2004).

2.1.1 *Nocardia*

An allergic pneumonitis called farmer's lung occurs among agricultural workers who have inhaled dust from moldy plant material. It has been traced to at least three actinomycetes – *Actinomyces*, *Nocardia* and *Streptomyces*. The most common organism in this group is *Nocardia* that is responsible to cause a variety of infections including Mycetoma. Sulfur granules may be seen by the naked eye in the specimen and under the microscope during direct microscopic examination of the specimen stained by H & E stain. In contrast to *Actinomyces*, species of *Nocardia* are inhabitants of the soil rather than commensals in animals and they are aerobic. *Nocardia* species are gram-positive and two species are pathogenic for man – *N. asteroides*, and *N. brasiliensis*, and are somewhat acid-fast. There are two common modes of infection by *Nocardia*, pulmonary nocardiosis arises from inhalation of the organisms, whereas chronic subcutaneous absesses (mycetomas) arise from contamination of skin wounds, usually on the feet, and hands of labourers. In pulmonary nocardiosis, the lesions may simulate miliary or pulmonary tuberculosis. *N. asteroides* lie scattered though the abscesses in the form of tangled, fine, branching filaments. Aggregations into granules do not occur. Different species of Nocardias are associated with mycetomas in different parts of the world, such as *N. brasiliensis* in Mexico. These abscesses are clinically very similar to those of

Streptomyces and to various fungi. *Nocardia* are widely distributed throughout temperate and tropical climates. The diseases it causes are seen frequently in association with immunosuppression or underlying chronic diseases such as Hodgkin's disease. Once nocardiosis becomes clinically evident, it tends to become progressive and fatal, even with aggressive therapy: about 50% of patients succumb. Various antibacterial drugs are used in the treatment of nocardiosis, and sulfonamides are reported to be most effective.

2.1.2 *Actinomyces*

Actinomycosis generally arise from endogenous inhabitants of the oral cavity, whereas nocardiosis results from inhalation of soil organisms. Several species of *Actinomyces* have been implicated as the cause of actinomycosis in humans and animals. *A. israelli* is usually responsible for disease in man. It is part of the normal oral flora. It can be cultured from the majority of human tonsils and is nearly always found in scrapings of gums and teeth. The conditions that lead the organism to become invasive are not definitely known but may involve trauma and dental surgery. In general, actinomycotic infections are accompanied by a mixed flora of gram-negative bacteria (actinobacillus, Eikenella, Fusobacterium, and Bacteroides). *Actinomycosis* is distributed worldwide but is relatively rare. Its incidence is higher in men than women and in persons over 20. *Actinomycosis* is characterised by chronic destructive abscesses of connective tissues; the abdomen (especially the caecum and appendix), the lungs, the chest wall, and the face and neck may be involved. Wherever the lesions occur, they are basically the same. Abscesses expand into contiguous tissues and eventually form

burrowing, tortuous sinuses to the skin surface, where they discharge purulent material. When pus from an abscess or infected sputum is examined carefully, yellow sulphur granules are occasionally seen. These are small colonies of actinomycetes, which may be up to several millimetres in diameter. Detection of granules is not required to establish a diagnosis of actinomycosis but their presence facilitates identification of the organism. Penicillin is the drug of choice. The organism is also sensitive to tetracycline.

2.1.3 *Streptomyces*

Streptomyces are characterised by the stability of their filaments and by the formation of spores on the aerial mycelia and by the formation of spores on the aerial mycelia that project above the surface of the culture medium. With increasing appreciation of the distinction between norcardiae and streptomycetes, it has been realised that both cause actinomycotic abscesses. Because streptomycetes are ubiquitous in soil, infection is attributed to contamination of scratches and penetrating wounds. Mycetomas caused by streptomycetes are indistinguishable clinically from other actinomycetes.

2.1.4 Microbial secondary metabolites from actinomycetes

Actinomycetes represent the microbial group richest in production of variable secondary metabolites. These mostly bioactive molecules are the end products of complex multi-step biosynthetic pathways. Recent progress in the molecular genetics and

biochemistry of the biosynthetic capacities of actinomycetes enables first attempts to redesign these pathways in a directed fashion. However, in contrast to several examples of designed biochemical improvement of primary metabolic processes in microorganisms, none of the products or strains derived from pathway engineering in actinomycetes discussed herein have reached pilot or production scale. The main reasons for this slow progress are the complicated pathways themselves, their complex regulation during the actinomycete cell cycle, and their uniqueness, as most pathways and products are specific for a strain rather than for a given species or larger taxonomic group (Piepersberg, 1994).

Microbial secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economics of our society. They often have unusual structures and their formation is regulated by nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction. Regulation is influenced by unique low molecular mass compounds, transfer RNA, sigma factors and gene products formed during post-exponential development. The synthases of secondary metabolism are often coded by clustered genes on chromosomal DNA and infrequently on plasmid DNA. Unlike primary metabolism, the pathways of secondary metabolism are still not understood to a great degree and thus provide opportunities for basic investigations of enzymology, control and differentiation. Secondary metabolism is brought on by exhaustion of a

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