UNIVERSITI PUTRA MALAYSIA

APPLICATION OF FUNCTIONAL METAGENOMICS FOR ISOLATION AND SCREENING OF ANTIMICROBIAL ACTIVITY OF ANTARCTIC SOIL MICROORGANISMS

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By

PREMMALA A/P RANGASAMY

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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August 2016

Chairman : Leslie Than Thian Lung, PhD
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Thousands of secondary metabolites have been identified from the culture of Gram positive, Gram negative and filamentous fungi that have been isolated from the Antarctic environment and since the year 2002. About 130 to 140 of these natural products have been used in human medicine, veterinary medicine and agriculture as pesticides. Bacteriocin production is also known as common ability among soil bacteria to outcompete competitor for resources including nutrient and water. Such ability is expected to be essential to the survival in the harsh environmental condition in Antarctica. In order to understand if Antarctic soils harbour novel bacteriocins with potential medical application, a functional metagenomic screening need to be conducted using the environmental DNA of the soil. Soil DNA extraction were done by using the Epicenter Meta-g-nome DNA extraction kit and the construction of metagenomic library were done by using the CopyControl Fosmid Library Construction kit from Epicenter. Two libraries were constructed and screened with different types of pathogens using the double agar layer method and incubated overnight at 37°C. Inhibition around the clones were observed the next day. Positive clones were picked for further screening. Positive clones were sent for Illumina next generation sequencing (NGS). The responsible gene that were identified is further cloned into an expression vector to confirm the production of antibiotic activity. Further screening were done by purifying the selected clone with C18 cartridge using the solid phase extraction method (SPE) with different percentage of solvent fractions. SPE products were evaporated using the microcentrifuge vapour evaporator at 30°C and were tested on the bacteria using the well diffusion method at the concentration of 350mg/ml. Inhibition zone were observed. By constructing two separate fosmid libraries which in total encompassed >15, 000 clones, 4 fosmid clones showing positive inhibition against *Klebsiella pneumoniea* were detected. BLAST results based on the DNA contigs generated using illumina NGS revealed that the clones carried periplasmic thiol-disulfide interchange protein DsbA gene and pathways related to isoprenoids for quinones and tyrosine, and phenylalaline

branches from chorismate. Virtual screening using Antibiotics and Secondary Metabolites Shell (Antismash) server corroborated our observation that the selected clones harboured bacteriocin production gene. We have attempted to purify the bacterial product using C-18 solid phase extraction and had obtained fraction (80% water, 20% methanol) which conserved the antibacterial effect against *Klebsiella pneumoniea*. The fraction was sent for a liquid chromatography – mass spectrometry (LCMS) analysis and it showed Nalidixic acid which could be the responsible metabolite. Further screening need to be done.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

APLIKASI METAGENOMIK BERFUNGSI UNTUK PENGASINGAN DAN PEMERIKSAAN AKTIVITI ANTIMIKROBIOLOGI DARIPADA MIKROORGANISMA YANG DIDAPATI DALAM TANAH

Oleh

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Beribu-ribu metabolit sekunder telah dikenal pasti daripada kultur Gram positif, kulat dan Gram negatif yang telah diasingkan daripada persekitaran Antartika dan sejak tahun 2002. Sehingga kini, 130-140 produk-produk semulajadi yang telah digunakan dalam perubatan manusia, perubatan veterinar dan pertanian sebagai racun perosak. Pengeluaran bakteriosin juga dikenali sebagai keupayaan biasa di kalangan bakteria tanah untuk bersaingan dengan pesaing untuk keperluan sumber termasuk nutrien dan air. Keupayaan tersebut dijangka menjadi penting untuk terus hidup dalam keadaan alam sekitar yang keras di Antartika. Untuk memahami jika tanah Antartika ialah pelabuhan untuk bacteriocins novel yang mempunyai potensi aplikasi dalam bidang perubatan, pemeriksaan metagenomic yang berfungsi perlu dijalankan dengan menggunakan DNA alam sekitar tanah. Pengekstrakan DNA dari tanah telah dilakukan dengan menggunakan Epicenter Meta-g-nome kit pengekstrakan DNA dan pembinaan perpustakaan metagenomic telah dilakukan dengan menggunakan kit CopyControl Fosmid Perpustakaan Pembinaan daripada Epicenter. Dua perpustakaan telah dibina dan ditapis dengan pelbagai jenis patogen menggunakan kaedah lapisan agar berganda dan dieram semalaman pada 37 ° C. Inhibisi sekitar klon dapat diperhatikan pada hari berikutnya. Klon positif dikumpul untuk pemeriksaan lanjut. Klon positif telah dihantar untuk Illumina Next generation sequencing (NGS). Gen bertanggungjawab yang telah dikenal pasti seterusnya diklon ke dalam vektor ungkapan untuk mengesahkan pengeluaran aktiviti antibiotik. Pemeriksaan selanjutnya dilakukan dengan memurnikan klon yang dipilih dengan kartrij C18 menggunakan kaedah pengekstrakan fasa pepejal (SPE) dengan peratusan yang berbeza pecahan pelarut. Produk SPE telah sejat menggunakan microcentrifuge wap penyejat pada 30 ° C dan telah diuji pada bakteria menggunakan kaedah penyebaran baik pada kepekatan 350mg / ml. Zon perencatan diperhatikan. Dengan membina dua perpustakaan fosmid berasingan yang secara keseluruhan meliputi> 15, 000 klon, 4 fosmid klon menunjukkan perencatan positif terhadap Klebsiella pneumoniea dikesan. Keputusan BLAST berdasarkan contigs DNA dijana menggunakan Illumina

NGS mendedahkan bahawa klon dijalankan periplasmic thiol-disulfida pertukaran protein DsbA gen dan laluan yang berkaitan dengan isoprenoids untuk quinone dan tyrosine, dan cawangan phenylalaline dari chorismate. Antismash menyokong pemerhatian kami bahawa klon terpilih memendam bakteriosin gen pengeluaran. Kami telah cuba untuk membersihkan produk bakteria menggunakan C-18 pengekstrakan fasa pepejal dan telah mendapat sebahagian kecil (80% air, 20% metanol) yang mempunyai kesan antibakteria terhadap *Klebsiella pneumoniea*. Pecahan telah dihantar untuk liquid chromatography – mass spectrometry (LCMS) analisis dan ia menunjukkan asid Nalidixic yang boleh menjadi metabolit yang bertanggungjawab. Pemeriksaan lanjut perlu dilakukan.

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I certify that a Thesis Examination Committee has met on 12 August 2016 to conduct the final examination of Premmala a/p Rangasamy on her thesis entitled "Application of Functional Metagenomics for Isolation and Screening of Antimicrobial Activity of Antarctic Soil Microorganisms" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Anti-SMASH	Antibiotics and secondary metabolite analysis Shell
ATCC	American Type Culture Collection
BAC	Bacterial Artificial Chromosomes
BLAST	Basic local alignment search tool
bp	Base pair
$CaCl_2$	Calcium chloride
CAMP	Cationic antimicrobial peptide
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
Dha	Didehydroalanine
Dhb	Didehydrobutyrine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleis triphosphate
Dsb	Disulfide bond
EDTA	Ethylenediaminetetraacetic acid
GCMS	Gas chromatography – mass spectrometry
GPS	Global positioning system
GST	Gluthathione transferase
HPLC	High performance liquid chromatography
LCMS	Liquid chromatography – mass spectrometry
LLE	Liquid – liquid extraction
LB	Luria – Bertani
Lan	Lanthionine
$MgCl_2$	Magnesium chloride
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaOH	Sodium hydroxide
NCBI	National Center of Biotechnology Information
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
OD	Optical density
ORF	Open reading frame

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Phage dilution buffer
PEP	Phosphenolpyruvate
RAST	Rapid annotation using subsystem technology
RE	Restriction enzyme
RNA	Ribonucleic acid
SDA	Sabouraud dextrose agar
SOC	Super optimal broth with catabolite repression
SPE	Solid phase extraction
SSDB	Sequence similarity data base
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TLC	Thin-layer chromatography
TRX	Thioredoxin
UTI	Urinary tract infection
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Antarctica is a continent almost completely covered by thick ice sheets. The functional roles of bacteria are particularly significant in Antarctica, where terrestrial trophic interactions are generally dominated by microorganisms (Chong et al., 2012; Hogg et al., 2006). It is almost twice the size of Australia, with regions of cold desert soils that have little free water and temperatures that rarely if ever rise above freezing. Antarctica is covered by ice that is on average at least two kilometers in thickness. It is the coldest, driest continent on Earth, and its habitats and organisms have little access to free water. Only during summer are its simple soil and rock habitats free from snow and ice (O'Brien et al., 2004).

The Southern Ocean is a broad physical barrier to the colonization of Antarctica by microorganisms and other biota, compounded by the lack of habitats suitable for their establishment. The islands and ice-free exposures of the sub-Antarctic and maritime Antarctic have somewhat more benign environmental conditions due to their warmer and moister climate, buffered by the surrounding ocean. Temperature, length of growing season and moisture availability play important roles in the colonization of these microorganisms (Smith, 1992).

Antarctic soils have fascinated researchers throughout the last century, being the focus of microbiological and other environmental studies (Pearce et al., 2012). As early as 1903, Elkelof found a significant numbers of bacteria in Antarctic soils, with greater numbers during summer (Boyd & Boyd, 1963).

Prokaryotes dominate many Antarctic ecosystems, where they play a major role in food chains, biogeochemical cycling of nutrients, and the mineralization of pollutants. Antarctica is an important example of an environment dominated by prokaryotes, playing important roles in trophic networks and biogeochemical cycles. Bacteria are very important members of trophic webs, and they are extremely adaptable enabling survival under a wide range of environmental conditions (Russell et al., 1990). There has been a focus on research on cold-adapted microorganisms, generally known as psychrophilic or psychtotolerant, which are capable of growth and reproduction at low temperature. They have specific modifications to their enzymes, membranes and other cellular components which allow them to function at low temperature (Russell et al., 2006).

Thousands of secondary metabolites have been identified from cultures of Gram-positive and Gram-negative bacteria, and filamentous fungi, that have been isolated from the Antarctic environment (Lewis et al., 2010). Since 2002, 130-140 of these secondary metabolites have seen application in human medicine, veterinary medicine and agriculture (Moncheva et al., 2002). Although the first commercialized

antibiotic, penicillin, was discovered by Alexander Fleming by chance, most present-day antibiotics are discovered and developed through systematic searching – 'bioprospecting'. The systemic screening path introduced by Paul Ehrlich has come to form the core of the search for new drugs in clinical practice and trials, and in the pharmaceutical industry generally (Aminov, 2010).

According to Crag et al. (1997), roughly 60% of the world's population depends almost completely on plants and natural products from various environmental sources such as water and soils that have been identified as an important source of therapeutically effective medicines. These natural products show biological activity in assays and are generally small molecules with drug-like properties which are capable of being absorbed and metabolized by the body. In order to counter the rapidly increasing challenge of multidrug resistant pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) (Enright et al., 2002), new antibiotics need to be developed and one of the richest sources is from the uncultured microorganisms of soil. Although many antibiotics are available today, the need remains for the discovery of new compounds to help solve urgent therapeutic challenges such as the continuing problem of the development of drug resistance and new infective properties amongst pathogens, both of which are encouraged by the widespread and often uncontrolled use of antibiotics (Gillespie et al., 2002).

The inability to culture most microorganisms obtained from the environment has frustrated microbiologists for decades (Daniel, 2004). One means of overcoming this challenge is provided by rapid advances in molecular biological approaches. One such advance is the development of the field of metagenomics, which involves the cloning and analysis of genomes without the need for culturing of the source microorganisms (Sabree et al., 2009). This new field offers a path to studying and investigating microbial communities as entire units without cultivating their individual members (Handelsman, 2004).

1.1 Hypothesis

Microorganisms cultured from sub-Antarctic soils using metagenomic screening will produce novel active antimicrobial compounds.

1.2 Objective

General Objective:
To screen and identify prospective compounds with antimicrobial activities from metagenomic library clones constructed from DNA found in sub-Antarctic soil.

Specific objectives

1. To construct a metagenomic library of DNA isolated found in sub-Antarctic soil.
2. To screen for clones which have antimicrobial activities.

3. To identify genes that are responsible for antimicrobial activities.
4. To sub-clone and assess the potential putative genes with antimicrobial activities.

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