ISOLATION AND CHARACTERISATION OF STRESS COMPOUNDS PRODUCED BY MARINE SPONGE BACTERIA

CHOW LAI THENG

INSTITUTE FOR TROPICAL BIOLOGY AND CONSERVATION
UNIVERSITI MALAYSIA SABAH
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CHOW LAI THENG

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DECLARATION

I hereby to declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been duly acknowledged.

03 March 2015

Chow Lai Theng
PP20098234

CERTIFICATION

NAME : **CHOW LAI THENG**

MATRIX NO. : **PP2009-8234**

TITLE : **ISOLATION AND CHARACTERISATION OF STRESS COMPOUNDS PRODUCED BY MARINE SPONGE BACTERIA**

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DECLARED;

1. **Supervisor**
   Prof. Dr. Charles Vairappan Tandatangan

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ABSTRACT

In order to cope with the imbalanced nutrient growth conditions, stress compounds are commonly synthesised by bacteria as the strategy of survival. Examples of bacterial stress compounds are polyhydroxyalkanoate (PHA), triacyglycerol (TAG) and wax ester (WE) inclusions. However, there has not been any prior report on marine stress compounds-producing bacteria isolated from sponges. Hence, present investigation looks into the isolation and identification of stress compounds-producing bacteria from marine sponge. Structural elucidation of the extracted stress compounds was also studied. In an attempt to isolate stress compounds-producing bacteria from marine sponge, *Halichondria* sp. and *Epipolasis* sp. were collected from Mantanani Island. *Halichondria* sp. leads to 15 isolates of marine bacteria, while *Epipolasis* sp. yielded a total of 42 strains of bacteria. One-step batch cultivation was performed for each of the strains. One bacterial strain, isolate HSP101R2-7 from *Halichondria* sp. and two strains from *Epipolasis* sp.: ESP101R3-6 and ESP101R2-12 were detected to possess the ability to accumulate stress compounds by phase contrast microscopy and Nile red staining. The effect of cultivation period and carbon sources was then evaluated by culturing the strains in the mineral salts medium (MM) with different types of carbon sources such as fructose, glucose, sucrose, fish cod liver oil (RCLO) and commercial fish cod liver oil (CFCLO). The incubation time was set to 48 hours, 72 hours and 96 hours. High yield of stress compounds as much as 3.6 mg/mL was extracted from isolate HSP101R2-7 isolated from *Halichondria* sp. This strain was identified as *Gordonia terrae* using Gram staining and 16S rDNA analysis. This is the first report on the isolation and identification sponge-derived bacteria with the ability to synthesise stress compounds under nitrogen-limitng growth condition. Nuclear magnetic resonance (NMR) was used to identify the structure of the stress compounds and TAG was confirmed as the only type of stress compound accumulated by *Gordonia terrae*. TAG inclusions appeared as spherical, clear-edged and surrounded by a layer of membrane based on the TEM micrograph. In addition, the purified TAG was subjected to gel permeation chromatography (GPC) and differential scanning calorimetric (DSC) analysis to study the material properties. Result showed that purified TAG has low thermal stability.

ABSTRAK

SINTESIS KOMPAUN-KOMPAUN TEKANAN DARIPADA BAKTERIA YANG BERASAL DARI SPAN MARIN

Untuk menyesuaikan ketidakseimbangan nutrien dalam pertumbuhan, kompaun tekanan biasanya akan disintesikan oleh bakteria sebagai strategi untuk terus hidup. Antara contoh kompaun tekanan yang disintesiskan oleh bakteria adalah seperti polihidroksialkanoat (PHA), triasigliserol (TAG), dan ester lilin (WE). Namun, sehingga kini tiada laporan saintifik mengenai tekanan bakteria marin penghasil-kompaun yang dipencilkan daripada span. Oleh itu, penyelidikan ini adalah untuk mengkaji pemencilan dan pengenalpastian tekanan bakteria penghasil-kompaun daripada span marin. Struktur kompaun-kompaun tekanan juga dikenalpasti. Dalam percubaan untuk memencilkan bakteria penghasil kompaun tekanan daripada span marin, Halichondria sp. dan Epipolasis sp. telah dikumpulkan dari Pulau Mantanani. Halichondria sp. telah menghasilkan 15 jenis bakteria marin, manakala Epipolasis sp. telah menghasilkan 42 jenis bakteria. Satu peringkat pengkulturan telah dijalankan bagi setiap satu bakteria tersebut. Satu sampel bakteria HSP101R2-7 dari Halichondria sp. dan dua jenis bakteria dari Epipolasis sp.: ESP101R3-6 dan ESP101R2-12 telah dikesan mempunyai keupayaan mensintesiskan kompaun tekanan melalui mikroskopi fasa terbalik dan pewarnaan Nile merah. Kesan jangka masa dalam inkubasi dan bekalan karbon telah dikaji di mana bakteria tersebut dikulturkan dalam medium garam mineral (MM) yang mengandungi pelbagai jenis sumber karbon termasuklah fruktosa, glukosa, sukrosa, minyak hati ikan kod (RCLO) dan minyak hati ikan kod komersial (CFCLO). Jangka masa pengkulturan telah ditetapkan hingga 48 jam, 72 jam dan 96 jam. Hasil kompaun tekanan yang tinggi sebanyak 3.6 mg/mL telah diekstrak dari bakteria HSP101R2-7 yang dipencilkan daripada Halichondria sp. Strain ini telah dikenalpasti sebagai Gordonia terrae dengan menggunakan pewarnaan Gram dan analisis 16S rDNA. Laporan ini merupakan penyelidikan pertama yang melaporkan pemencilan dan pengenalpastian bakteria yang berasal dari span yang berupaya mensintesiskan kompaun tekanan di bawah persekitaran pertumbuhan sumber nitrogen adalah terhad. Nuklear magnetik resonansi telah digunakan untuk mengenalpasti struktur bagi kompaun tekanan dan TAG telah dikenalpasti sebagai satu-satunya kompaun tekanan yang disintesiskan oleh Gordonia terrae. TAG dikenali dengan berbentuk sfera yang mempunyai sisi jelas dan dikelilingi satu lapisan membran berdasarkan TEM mikrograf. Di samping itu, TAG telah dikaji melalui gel penyerapan kromatogrfi (GPC) dan pembezaan imbaan kalorimetri (DSC) untuk mengkaji sifat-sifat fizikal dan mekanikal. Keputusan menunjukkan TAG mempunyai kestabilan haba yang rendah.

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LIST OF ABBREVIATIONS

^{1}H-NMR	-	proton nuclear magnetic resonance
^{13}C-NMR	-	carbon-13 nuclear magnetic resonance
CFCLO	-	commercial fish cod liver oil
DCW	-	dry cell weight
DSC	-	differential scanning calorimetric
GPS	-	global positioning system
Kb	-	kilobit
KCl	-	potassium chloride
KH_2PO_4	-	potassium hydrogen phosphate
M	-	Molarity
$MgSO_4$	-	magnesium sulphate
MHz	-	mega hertz
MM	-	nitrogen-limiting mineral medium
NaCl	-	sodium chloride
Na_2HPO_4	-	disodium phosphate
$NaSO_4$	-	sodium sulphate
NH_4Cl	-	ammonium chloride
Ng	-	nanogram
Nm	-	nanometers
NMR	-	nuclear magnetic resonance
NR	-	nutrient rich
PHA	-	polyhydroxyalkanoate
RCLO	-	stingray cod liver oil
Rpm	-	revolutions per minute
Scuba	-	self-contained underwater breathing apparatus
Sp	-	species
TAG	-	triacylglycerol
UV	-	ultra violet
v/v	-	volume per volume
WE	-	wax ester

wt%	-	weight percentage
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Bacteria and Bacterial Stress Compounds

Bacteria are a group of prokaryotes or known as unicellular microorganisms, which could not be seen through naked eyes. Although bacteria are microscopic, major portion of the world's biomass are dominated by them. They are ubiquitous in a wide variety of habitats ranging from the coldest Antarctica to hot springs. And even more extreme harsh condition, certain bacteria, for example *Citromicrobium bathyomarinum* Yurkov can survive in the salty deep oceans (Yurkov *et al*., 1999). What has enabled the successful survival of bacteria is their capability to reproduce rapidly under optimal conditions. In many species, cell division occurs every twenty minutes. Bacteria have short life cycle, but surprisingly their adaptability to various environmental changes is way more efficient than the eukaryotic organisms.

The discovery of bacteria has raised the question such as what are the advantages and disadvantages of these unique prokaryotic creatures. Usually, most people are aware of bacteria by associating them with some negative perceptions - infectious diseases such as pneumonia, tuberculosis and chorela. The vast majority of bacteria are not pathogenic, in fact they are important for human health. For instance, *Escherichia coli* Migula, a common bacterium found in the large intestines of human helps in the synthesis of vitamin K and vitamins B. In industry, bacteria show a great potential in the production of food through fermentation, treatment of sewage, bioremediation of oil spill and the synthesis of vitamins and antibiotics.

The bacterial growth and physiological responses to environmental conditions are unique. In most cases, the growth rate of bacteria is characterized by several aspects such as nutritional factor, solute concentrations, oxygen levels, growth temperature and pH (Moat *et al.*, 2002; Serrazanetti *et al.*, 2011). Alterations in any of these mentioned parameters may contribute to the production of microbial stress compounds. In order to cope with the stressful conditions, some microorganisms may synthesise specific types of stress compounds via various metabolic pathways. For bacteria, stress compounds occur as bacterial inclusions in cytoplasm. Cyanophycin inclusions, glycogen inclusions, metal inclusions, sulfur inclusions, polyphosphate inclusions, wax ester (WE) inclusions, polyhydroxyalkanoate (PHA) inclusions and triacyglycerol (TAG) inclusions are some examples of bacterial inclusions (Alvarez and Steinbüchel, 2002; Shively, 2006). And these bacterial inclusions are solid chemical compounds, which can be inorganic such as polyphosphate and sulfur or organic like carbohydrates, lipid and peptide. Several reports reviewed that PHA, TAG and WE were the stress compounds accumulated by bacteria as the strategy of survival under unfavourable growth conditions. For an example, during the depletion of essential nutrient such as phosphorus, nitrogen and magnesium, some bacteria are capable in producing PHA to serve as carbon and energy storage compound (Lugg *et al.*, 2008).

Polyhydroxyalkanoate (PHA) is usually known as biodegradable plastics or microbial plastics. PHA is reserve polyesters that can be intracellularly synthesised by numerous microorganisms such as eubacteria and Archea under different nutrient and environmental conditions (Du *et al.*, 2001; Rehm, 2003; Alias and Tan, 2005; Jendrossek, 2009). For bacteria, PHA is a high-energy bacterial stress compound. Bacteria may deposit PHA when the nutrient supplies such as phosphorus, nitrogen, sulphur, magnesium or oxygen are limited (Pantazaki *et al.*, 2009; Keshavarz and Roy, 2010; Lau *et al.*, 2010). According to Luengo *et al.* (2003), these polymers commonly appear in the form of lipid in bacterial cell cytoplasm with the function as carbon and energy storage reservoirs. However, the types of accumulated PHA with different monomeric composition, macromolecular structure, number and size of granules, physical properties, and chemical properties vary according to the microbes. Hence, it draws the attention of scientists towards the technology of biopolymer.

Initially work on PHA was carried out by Maurice Lemoigne in 1926. He discovered that poly(3-hydroxybutyrate) P(3HB) was able to be stored by the Gram-negative bacteria, *Bacillus megaterium* de Bary (Luengo *et al.*, 2003; Chien *et al.*, 2007; Keshavarz and Roy, 2010). Later, a wide range of Gram-positive and Gram-negative bacteria were found capable in accumulating PHA as intracellular granules. In current research, several bacteria were reported to synthesise PHA which acts as a carbon and energy reservoir up to 90% of their cellular dry matter under starvation condition (Reddy *et al.*, 2003).

Meanwhile, triacylglycerol (TAG) is a simple lipid class which widespread in the eukaryotic cells. For eukaryotic organisms, TAG is vital considering it serves as the storage form for energy and fatty acids, which involved in membrane biosynthesis (Sorger and Daum, 2002). Yen *et al.* (2005) and Daniel *et al.* (2011) correspondingly postulated that TAG is actively synthesised in adipose tissues, liver, intestine and mammary glands of mammals. As for plants, TAG is accumulated in seeds (Daniel *et al.*, 2011; Santala *et al.*, 2011). Besides, TAG also can be traced even in the simple eukaryotic microorganisms like fungi and yeast (Sorger and Daum, 2002).

Unlike the eukaryotic organisms, TAG is hardly produced by the prokaryotes. Only a few groups from the prokaryotic organisms such as actinomycetes and some of the human pathogens possess the capability to accumulate TAG as a storage compound (Christie, 2011). TAG is one of the stress compounds synthesised by the bacteria in response with limitation of nitrogen and oversupply of carbon source (Alvarez and Steinbüchel, 2002). According to Nakagawa *et al.* (1976), the mycobacterial pathogen – *Mycobacterium smegmatis* Trevisan tends to deposit TAG in the bacterial cells during the late stage of cell growth under starved conditions. Some bacteria were reported capable of producing TAG as much as 80% cell dry mass when dealing with environmental stress (Manilla-Pérez *et al.*, 2010; Santala *et al.*, 2011). Overall, TAG occurs as cytoplasmic lipid inclusion bodies within both the eukaryotes and prokaryotes.

Wax ester (WE) on the other hand is another type of bacterial stress compound which presents in the form of lipid inclusions. Likes the TAG, WE is normally deposited by the eukaryotes. It is a major component to plant due to its hydrophobic property. WE is considered as a good water resistant layer because it able to prevent water loss from the leaves and fruits effectively. Meanwhile, WE can be located in sperm whale and it is important in controlling the buoyancy of sperm whale. Compare to the deep ocean fish, WE in other animals and insects secretions works as a protective coating (Wahlen *et al.*, 2009).

So far, only a few species of bacteria were found capable to accumulate WE intracellularly. For instance, WE is the common storage material for species of bacteria from genus *Acinetobacter* (Alvarez *et al.*, 1997; Rontani *et al.*, 1999; Manilla-Pérez *et al.*, 2010). Also, *Marinobacter*, *Mycobacterium* and *Rhodococcus* will produce WE when dealing with limited nutrient such as nitrogen and phosphorus (Holtzapple and Schmidt-Dannert, 2007). In some cases, certain bacteria are able to synthesise more than one kind of lipid inclusions. For an example, the formations of both TAG and WE can be traced at the same time in cytoplasm of *Acinetobacter baylyi* Brisou and Prévot (Alvarez and Steinbüchel, 2002).

Besides the lipid inclusions, bacteria may produce other forms of stress compounds as the strategy of survival in response to stressful growth environment. For instance, one study has been published in which the lactic acid bacteria (LAB) will release alcohols, amino acid metabolites, aldehydes, esters, lactones and keto acids when facing with the nutritional stress environment (Barry and Wainwright, 1997; Serrazanetti *et al.*, 2011). Barry and Wainwright (1997) also reported on the synthesis of secondary metabolites, which were another kind of accumulated bacterial stress compounds during imbalanced growth conditions. And these secondary metabolites were found exhibiting great antibacterial activity.

1.2 Scope of Study

To investigate the diversity of stress compound-producing bacteria isolated from two different species of marine sponges, *Halichondria* sp. and *Epipolasis* sp. These sponges were collected from Mantanani Island. Mantanani Island is located at the Northern Coast of Sabah. In this study, PHA, TAG and WE were the targeted stress compounds.

1.3 Research Rationales

During the past two decades, there has been a growing interest for the study of bacterial stress compounds. Huge numbers of research activities have been focused on the stress compounds-producers isolated from the terrestrial environment. Studies of the biosynthesis of PHA have revealed the potential to accumulate PHA from glucose of five wild type strains, which isolated from ammunition-polluted environment. These five strains were *Bacillus anthracis* Cohn, *Bacillus mycoides* Flügge, *Bacillus pseudomycoides* Nakamura, *Bacillus thuringiensis* Berliner, and *Bacillus weihenstephanensis* Lechner et al. (Mizuno *et al.*, 2010). They were able to accumulate a high content of PHA ranging from 15 wt% to 44 wt%.

Both TAG and WE from eukaryotic organisms are of major interest due to their availability. Conversely, not much of studies have been carried out on the microbial TAG and WE. So far, few authors have reported that TAG biosynthesis was detected in human pathogenic bacteria and some actinomycetes. For example, three pathogenic strains were found to possess the ability to produce a high content of TAG under stress growth condition. These three strains belonged to *Mycobacterium* genus which includes *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* and *Mycobacterium phlei* (Nakagawa *et al.*, 1976; Daniel *et al.*, 2011). Meanwhile, it has been reported that one WE-producing bacterium, *Acinetobacter baylyi* was isolated from soils (Santala *et al.*, 2011).

Other than the terrestrial environment, marine microbial mat was used as the source for isolation of PHA-producing bacteria (Lȯpez-Cortés *et al.*, 2008). It has been reported that *Pseudomonas guenzennei* which isolated from microbial mats growing on Polynesian atolls also possesses the ability to accumulate PHA by using glucose as the carbon source (Simon-Colin *et al.*, 2008a, 2008b). To date, there are no reports on the production of PHA, TAG or WE by marine bacteria especially those isolated from sponge. Based on the available information, marine sponges are capable in maintaining numerous bacteria as they feed by filtering bacteria from water (Bhamrah and Juneja, 1999; Kanagasabhapathy *et al.*, 2005). Most researchers have taken an important step in understanding of antimicrobial activity of marine bacteria associated with sponge rather than isolation of stress compound-producing bacteria from marine sponge (Müller, 2003; Anand *et al.*, 2006; Kennedy *et al.*, 2009). Thus, the study of the capability of wild type marine bacteria to grow and produce stress compounds is essential.

1.4 Research Objectives

The objectives of this investigation were:

a. To isolate marine bacteria from two species of marine sponges.

b. To screen for stress compound-producing marine bacteria. Targeted stress compounds are mainly PHA, TAG and WE.

c. To identify isolated bacterial strains with the ability to synthesise stress compound.

d. To determine the monomeric composition of the stress compound obtained from marine bacteria via spectroscopic techniques.