Faculty of Resource Science and Technology

ISOLATION AND CHARACTERIZATION OF DELTAMETHRIN-RESISTANT BACTERIA ISOLATED FROM SOIL ORIGINATING FROM A PEPPER PLANTATION

Oliver Swenson Ak Ragib
19658

Bachelor of Science with Honours
(Resource Biotechnology)
2010

ISOLATION AND CHARACTERIZATION OF DELTAMETHRIN-RESISTANT BACTERIA ISOLATED FROM SOIL ORIGINATING FROM A PEPPER PLANTATION

Oliver Swenson Ak Ragib
19658

This project is submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

Department of Molecular Biology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2010

ACKNOWLEDGEMENT

First of all I want to thank God for His love and spiritual blessings upon me in completing this final year project. I would like to express my gratitude to my supervisor, Prof. Dr. Mohd. Azib Salleh for his advice, knowledge, and precious time he put for being a mentor and reviewing in my write-up. I would also want to express my appreciation to my fellow colleague Ms. Mazidatul Ashiqeen, Mr. Jakaria, Mr. Taufik and Ms. Ekin, my Molecular Genetics Lab Master student, Mr. Geoge Deng and Mr. Frazer, fellow friends and not to forget our lab assistant Mdm Sheela Ungau for their advice, guidance and friendship. Last but not least, I want to thank my mother, Mdm Lemik Ngagat and my family for their love and continuous moral and financial support throughout the period of completing this project. God bless you all.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

As – Arsenic

Cd – Cadmium

Co – Cobalt

Cu – Copper

Hg – Mercury

KAc – Potassium acetate

LB broth – Luria-Bertani broth

MHA – Mueller-Hinton agar

MIC – Minimal inhibitory concentration

Mn – Manganese

NA – Nutrient agar

NaOH – Sodium hydroxide

Pb – Lead

ppm – part per million

pytH gene – Pyrethroid-hydrolyzing carboxyesterase gene

SDS – Sodium dodecyl sulphate

Zn – Zinc

2,4-D – 2,4-dichlorophenoxy acid

ISOLATION AND CHARACTERIZATION OF DELTAMETHRIN-RESISTANT BACTERIA ISOLATED FROM SOIL ORIGINATING FROM A PEPPER PLANTATION

Oliver Swenson anak Ragib

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Sixteen bacterial strains were isolated from soil taken from a pepper plantation that have the history of deltamethrin application were analyzed. Five Gram-negative, rod strains were assessed for resistance profile against the ascending deltamethrin concentration and all strains were putatively identified to five different genera include *Acetobacter* spp., *Pseudomonas* spp., *Acidiphilium* spp., *Aminobacter* spp. *and Aeromonas* spp. *Aminobacter* spp. was identified as the prolific deltamethrin-tolerant bacteria among five strains, which is able to tolerate deltamethrin with concentration up to 9600 ppm. The multi-resistant ability of isolates was further assessed with the testing the tolerance of these bacteria toward three heavy metals, mercury, copper and zinc. All five isolate showed tolerance to at least one heavy metal, which is copper. *Aminobacter* spp. showed resistance toward all three heavy metals, which proved to be a suitable candidate for biodegrader in co-contaminated soil environment. No plasmid was detected in all five isolates. Therefore it is believed that the resistance ability of isolates toward deltamethrin and some heavy metals are chromosomal encoded enzymatic reaction.

Key words: Deltamethrin resitance, Heavy metal resistance, Multi-resistance ability, Plasmid

ABSTRAK

Sebanyak enam belas strain bacteria telah dipencil daripada sampel tanah kebun lada hitam yang telah lama terdedah dengan penggunaan deltamethrin. Lima strain Gram-negatif berbentuk rod telah diuji tahap kerintangan terhadap kepekatan deltamethrin secara mendadak. Semua strain tersebut telah disangka tergolong dalam genera *Acetobacter* spp., *Pseudomonas* spp., *Acidiphilium* spp., *Aminobacter* spp. dan *Aeromonas* spp.. Daripada kelima-lima strain, *Aminobacter* spp. telah dikenalpasti mempunyai daya rintang yang tertinggi, iaitu mampu hidup dalam kepekatan sehingga 9600 ppm. Sifat serba rintang dalam kelima-lima strain dikaji lebih lanjut dengan menguji kerintangan bakteria ini terhadap 3 logam berat iaitu merkuri, kuprum dan zink. Kesemua strain menunjukkan daya rintang sekurang-kurangnya satu jenis logam berat, iaitu kuprum. *Aminobacter* spp. menunjukkan daya rintang terhadap ketiga-tiga logam berat, dimana ini membuktikan strain ini merupakan calon yang paling sesuai untuk proses biodegradasi tanah yang mengandungi banyak agen pencemar. Tiada plasmid dikesan daripada kelima-lima strain. Oleh itu, daya rintang bakteria-bakteria ini terhadap deltamethrin dan logam berat adalah disebabkan mekanisma enzim yang dikod oleh DNA kromosom.

Kata kunci: Kerintangan terhadap deltamethrin, Kerintangan terhadap logam berat, Kepelbagaian daya rintang, Plasmid

INTRODUCTION

Modern society has introduced a large number of xenobiotics to the environment. Some of these compounds are readily degraded in the environment, whereas others are recalcitrant. Due to this fact, the interest in remediating and conserving the environment's natural biota has greatly increased in the past decades. Biological approaches in remediating xenobiotics have shown great potential in performing a task that promises no other side effects to the environment as what chemically approaches do. The term 'biodegradation' refers to the process of using microorganisms either in immobilized form or transformed environmental contaminants to simple end products (Glazer & Nikaido, 1995).

Malaysia is blessed for its fertile soil and is suitable for the plantation of wide varieties of crop. As a result of vast development in agricultural activities, the effects of pesticide utilization also takes place. Black pepper (*Piper nigrum* L.) has contributed significantly toward the economy and sosioeconomics of Sarawak. However, pepper cultivation encounters some obstacles that affect its growth, yield and longevity. The most serious constraint in pepper cultivation is the disease problem, which are commonly due to pests. Infection caused by insects have become a common threat as a number of insect pests have been identified such as aphid, red flour beetle, *Tribolium castaneum*, stem borer, *Lophobaris piperis*, green pepper berry bug, *Dasynus piperis*, and bug *Diconoris hewetti* (Wiratno, 2008). Therefore, commercially available pesticides have been used by farmers to protect their crops.

The main concern regarding xenobiotics dispersion in the environment is how the toxic compound may affect the health of people and contamination of the soil and aquatic environments. Pesticides enter the water reservoir below the ground particularly through surface runoff and leaching. In order to reduce the amount of xenobiotics from leaching into groundwater or directly to the water environment, the utilization of indigenous microorganisms originating from the soil is the most efficient approach.

Numerous studies have been conducted about the resistance and degradation of pesticides by microorganisms, both in pure and mixed culture systems. Microbial strains belonging to the genera *Pseudomonas, Bacillus, Xanthomonas* and *Rhodococcus*, as well as fungi have demonstrated high degradation capacities over a wide number of xenobiotics, including pesticide (Lopez *et al.*, 2005). Microorganism's biotransformation ability has shown a very specific process and it is most likely due to the enzymatic activity, which every indigenous bacterium possesses by adaptation to the pesticide.

The involvement of enzymatic activity signals for the involvement of genetic materials that produce them. Plasmid molecules play important roles in bacterial resistance and degradability of xenobiotics. Many resistance and catabolic genes are located on plasmid which are self-transmissible and have a broad host range (Sayler *et al*., 1990). The objective of this study was to isolate and characterize deltamethrin insecticide-resistant bacteria found in soil taken from pepper plantations. The present work was also aimed to screen for the presence of plasmid DNA that might be responsible for the resistance profile of bacterial isolates towards co-contaminating deltamethrin and heavy metals.

LITERATURE REVIEW

2.1 Background of pepper (*Piper nigrum* L.)

Piper nigrum L. is the scientific name for pepper which belongs to the family of piperaceace consisting of 17 Piper species. Pepper is the world's most sought after spice for food flavoring purpose. Today, pepper is one of most important commercial crops that Sarawak. The state produced 14,361 tonnes of pepper in 2008 which was valued at RM 118.3 million (Paulus, 2008).

2.2 Pests and diseases

Pest and disease are the main limiting factor in pepper production. A severely attacked pepper plant would have reduced vitality, hardly produce berries and will eventually die. The major pests manifestations are either attack the plant underground or above ground. According to Wiratno (2008), the most important insect pests attacking the upper part of pepper plants are the stem borer, *Lophobaris piperis*, green pepper berry bug, *Dasynus piperis*, and bug *Diconoris hewetti*. Stem borer is a small pepper weevil of which the larvae bore holes in the stems of the pepper plants. This will impair the climbing stem which finally the young plant will be hampered. On the other hand, the tinged bug is a large pepper berry bug that causes immature berries to fall down. As a result, huge losses were experienced by many black pepper planters. Unfortunately, all the cultivated varieties in Sarawak are also susceptible to Phytophthora foot rot disease that caused by fungi, *Pyhtophthora capsici* (Paulus, 1993). As a result of the high susceptibility toward disease, protecting pepper vines

against various pests and diseases, largely by chemical means have introduced unintended pollution to the surrounding biota.

2.3 Pyrethroid insecticide

Insecticides consist of a large group of chemicals that are used in agriculture and residential compound to control plant and animal infestation. In pepper plantation, pyrethroid insecticide is the most common pest controlling method compared to other pesticides. Pyrethroids are known for their quick "knock-down" effect that other insecticides lack.

There are two types of pyrethroid available, namely natural and synthetic pyrethroid. Due to the instability of natural pyerethroid in insect's elementary canal, it is proven to be unable to kill insects (Qureshi, 2005). Hence, the development of synthetic pyerethroid by the attachment of the alcohol to the dichlorovinyl derivatives of cyclopropanecarboxylic acid moiety gives light and oxygen stability. In addition, the attachment of α-cyano group to the 3-phenoxybenzylalcohol moiety has enhanced the toxicity (Litchfield, 1983). Therefore, synthetic pyerethroids deltamethrin, permethrin, and cypermethrin are preferred insecticides as they are highly toxic to insects and low toxic to mammals. However, synthetic pyrethroids are extremely toxic to the aquatic environment, with only 10 ng L^{-1} being enough to eradicate all invertebrate life in whole rivers and lakes (Pearce, 1997).

2.4 Mechanism of microbial resistance to deltamethrin

Microorganisms especially bacteria that continuously exposed to the environmental stresses have shown development in their genetics against toxicants (Parsek *et al.*, 1995). The ability of indigenous bacteria to tolerate xenobiotics in its environment is strongly affected by its ability to utilize the xenobiotics itself (Summers, 1996). Many genera of bacteria have been isolated from different parts of the world with unusual properties to degrade xenobiotics contaminants. These include the genera *Pseudomonas* sp., *Arthrobacter* sp., *Ralstonia* sp. and *Rhodococcus* sp. (Noordman & Janssen, 2002); *Serratia* sp., *Bacillus* sp., and *Klebsiella* sp. (Rani *et al.*, 2008), *Xanthomonas* sp. (Lopez *et al.*, 2005) and *Sphingobium* sp. (Wang *et al.*, 2009).

At molecular level, there are three common mechanisms of resistance of bacteria against pesticides that are present in its environment, namely by changing the target molecules, inactivation of the functional group, and the sequestering the pesticide. The inactivation of the functional group of pyrethroid is an enzymatic process which involves pyrethroid hydrolase (Wang *et al.*, 2009). In Maloney *et al*. (1988) review, the principal mode of pyrethroid decomposition includes hydrolysis of ester bonds and oxidation of acid and alcohol functional groups. Esterase hydrolyzes the ester linkage between the acid and the alcohol moieties. As a result, one of the major products, 3-phenoxybenzoic acid will be further transformed into 4-hydroxy-3-phenoxybenzoic acid, which is a non-toxic compound and thus inactivate the insecticidal property.

2.5 Gene involved in the resistance toward deltamethrin

The latest documented gene that encodes pyrethroid hydrolase is the pyrethroid-hydrolyzing carboxyesterase (*pytH*) gene (Wang *et al.*, 2009). *pytH* gene is a chromosomal DNA and was isolated from *Sphingobium* sp., which known to capable of degrading wide range of pyrethroid insecticide. The nucleotide sequences of the *pytH* genes of *Sphingobium* sp. strain JZ-1 were deposited in the GenBank database under accession number FJ688006.

2.6 Heavy metals

Heavy metals are defined as elements which have a density greater than 6 g cm^{-3} (Naidu *et al.*, 2001). There are two types of heavy metals, namely the essential and non-essential heavy metals. The essential heavy metals include cobalt (Co), copper (Cu), manganese (Mn) and zinc (Zn). These are trace elements, also known as micronutrients, are required in low concentrations for biological processes for most organisms. The non-essential heavy metal includes cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As). These non-essential elements are toxic to plants, animals and microorganisms.

Heavy metals in terrestrial and aquatic environments either originated from natural sources or anthropogenic activities (Naidu *et al.*, 2001).Natural sources origin can be explained through soil formation. Soil formation is influenced by the parent rock materials, time, climate and organisms. Thus, the concentration of naturally occurring heavy metals released during soil formation process is related to the origin and nature of parent material. In contrast, anthropogenic source of heavy metals include industrial processes, mining and agricultural practices (Alloway, 1995).

2.7 Heavy metal toxicity to microorganisms

Trace amounts of essential heavy metals may be essential for the physiological balance in microorganism. For example, microorganisms require trace amount of copper as protein cofactor (van der Lelie & Tibazarwa, 2001). However, the occurrence of heavy metal contamination in soil and effluent, potential threat is presented as microbial populations differ in sensitivity to heavy metal (Giller *et al.*, 1998). The presence of heavy metals also can inhibit a broad range of microbial processes, including the dehalogenation process, which is essential to the mineralization of many organic pollutants in soil (Sandrin & Maier, 2003). However, there are studies on the isolated heavy metal tolerant bacterial species such as *Staphylococcus* sp., *Salmonella* sp., *Bacillus* sp. and *Pseudomonas* sp. (Olukoya, Smith & Illori, 1997); *Klebsiella pneumonia* (Karbasizaed, Badami & Emtiazi, 2003) which can survive in highly metal contaminated soils.

2.8 Relationship between xenobiotic resistance capability and resistance to heavy metal.

Indigenous bacteria that show resistance towards certain xenobiotics have been shown to be due to the involvement of enzymatic metabolism that inactivates the functional group of xenobiotic (Parsek *et al.*, 1995). Heavy metals have been reported to show inhibiting and stimulating effect to the enzymatic process of bacterial degradation of organic pollutant (Sandrin & Maier, 2003). The study also showed the relationship between the concentration of heavy metal in the soil and the ability of bacteria to degrade xenobiotics. As expected, the

inhibition increases progressively as the concentration of bioavailable metal in a co-contaminated environment increases.

However, as a result of the huge diversity of soil microbiota and also the co-existence of heavy metals, another pattern of metal effect on biodegradation has been identified. According to Capone *et al.* (1983), different metal toxicity in which low metal concentrations stimulate biodegradation activity whereas high metal concentration inhibits biodegradation. It is suggested that the differential inhibition of the methanogenic and non-methanogenic microorganisms. Metals may have selected for a metal-resistant, methanogenic population, and suppress nonmetal-resistant population.

Another pattern of heavy metal effects on microbial biodegradation shows high metal concentration tend to stimulate biodegradation while low metal concentration inhibit biodegradation (Roane & Pepper, 1997). High cadmium concentration create selective pressure for metal-resistant, 2,4-dichlorophenoxy acid (2,4-D) degrading microorganisms. This selective pressure may have reduced competition from metal-sensitive, non-degrading microorganisms, thus increasing biodegradation at higher metal concentrations.

2.9 Plasmid DNA

Plasmids are extrachromosomal circular DNA fragments that ranges in size from 1 to >200kb. Plasmid DNAs are transferable from one bacterium to another, and whose replication occurs independently within the bacterial chromosome. For this reason, plasmid DNA replication is not linked to the processes of chromosomal replication and cell division. In many cases, plasmids are quite small, just a few kilobases in length. But in some organisms, for example member of genus *Pseudomonas,* plasmids of up to several hundred kilobases are common (Dale, 1989).

Although considered as a non-essential addition to genetic information of the host bacterium, the functions of plasmid have been identified to as a survival mechanism for bacterium possessing it in a typical environment. There are five primary types of plasmids with different function, namely fertility plasmid, resistance plasmid, col-plasmid, degradative plasmid and virulence plasmid (Phillips & Funnel, 2004). In environmental biotechnology, degradative plasmids are crucial for the indigenous properties which give these specific bacterial strains a significant role in its survival and indirectly maintaining the balance of the ecosystem. Degradative plasmids carry genes that confer on the host bacteria the ability to degrade recalcitrant organic compounds not commonly found in nature (Phillips & Funnel, 2004).

There are several advantages that linked to the characteristics of plasmid towards the fitness it brought to the host bacterium. Firstly, the stable inheritance at cell division of a bacterial plasmid implies that there is an efficient mechanism to ensure that each daughter cell receives at least one copy of the plasmid in cell division. Both types of bacteria which possess

high copy number of plasmid and another one is with low copy number (possessing conjugative F plasmid) transfer their plasmid by partitioning randomly between two daughter cells and conjugation, respectively. Secondly, plasmid shows high copy number of its multiplication. This characteristic has played an important role in resistance among bacteria toward stressor such as xenobiotics and antibiotics from one generation to another. Thirdly, the cell to cell plasmid transfer that favored by natural selection will cause it to spread rapidly (Summers, 1996). Cell to cell transfer by mean of conjugation, transduction and transformation may exhibit both pro and con effects. The resistance and degradative profile of indigenious bacteria toward contaminants in environment may be significantly beneficial. However, cell to cell transfer is always a threat to human health as the rapid resistant property of an antibiotic resistant bacterium will inevitably limit the availability of antibiotic resources.

With the rising attention towards the potential of plasmids, several plasmid isolation procedures have been reported. Most of the methods depend on the plasmid's size in comparison with the bacterial chromosome and on the circular form (Hardy, 1981). There are two commonly used methods, namely dye-buoyant density centrifugation and alkaline lysis extraction. To date, various commercially available plasmid DNA extraction kits are available for rapid and efficient purification of high copy and low copy plasmid DNA without the need precipitation and organic extractions.

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sample collection

Soil samples were taken from a pepper plantation located at Tapah, near to Serian district. To increase the variety of microflora in the soil, approximately a total of 500 grams of fresh soil was taken from 3 different spots in the farm. To obtain the soil sample, a sterilized spatula were used to dug approximately 5 cm into the earth around the "foot" (lower part of the stem) and were collected into a sterilized bottle. Prior to begin the experiment, the soil sample was kept in 4 °C in order to maintain the integrity of soil and its microbial content.

3.1.2 Growth medium

There are three most frequently used media for this project. Table 1 shows the manufacturer and the composition of each medium.

Table 1: Manufacturer and composition of media used in this project

Media	Manufacture	Composition (Per liter)
Nutrient agar (NA)	Oxoid, England	'Lab-Lemco' powder (1.0); Yeast extract (2.0); Peptone (5.0); Sodium Chloride (5.0); agar (15.0); pH 7.4
Mueller-Hinton agar (MHA)	Oxoid, England	Casein hydrolysate (17.5); Meat extract (4.0); Starch (1.5); Agar (15.0); pH 7.4
Luria Bertani broth	Difco, USA	Pancreatic digest of casein (10.0); Yeast extract (5.0); Sodium chloride (0.5); pH 7.0

Prior of use, the prepared media was autoclaved at 121°C, 15 psi and for 15 minutes. The supplementing of media with insecticide or heavy metal stock solution was done only the autoclaved media cools down to approximately 55°C. The mixture was mixed thoroughly prior to pour plating into sterile petri dish.

3.1.3 Working culture

The working culture of bacterial isolates was kept in slant agar of NA in Bijou bottle. The preparation was done by taking the single colony that grows on agar plate containing certain concentration of insecticide. The cultures were incubated overnight at 37°C and stored at 4°C.

3.1.4 Maintenance of pure bacterial culture (stock culture)

Pure cultures were maintained on nutrient agar slants at 4°C. Slant agar stock cultures were prepared by streaking a single colony onto the slant agar surface. By using sterilized inoculating loop, a quadrant streak was made. The cultures were then incubated at 37°C for 24 hours prior to storage in 4°C.

Glycerol stock culture was done at the end of the project. Bacterial strains with desirable characteristics were cultured for long term storage. A total of 1.5 ml mixture of overnight broth culture and 60% sterilized glycerol was stored in a 1.5 ml centrifuge tube and mixed upon storage in -20°C.

3.1.5 Control bacterial strain

Two different strains of bacteria as control were used in the experiment. The first strain is *Escherichia coli* strain EDL 433 (provided by Molecular Genetics Lab); whereas another one is *Pseudomonas aeruginosa* (provided by Microbiology Lab), which were used for screening and biochemical test, respectively.

3.1.6 Chemicals

Commercial grade deltamethrin insecticide (1.4% w/w active compound) manufactured by Zagro Chemicals Sdn. Bhd. were used to supplementing growth media (Nutrient agar) for screening for deltamethrin resistant bacteria.

3.1.7 Heavy metals

Copper sulphate ($CuSO_4$), Mercury chloride ($HgCl_2$) and Zinc chloride ($ZnCl_2$) salts were used to prepare copper, mercury and zinc cations, respectively.

3.1.8 Stock solution

For every experimental session, the stock solutions of insecticide and heavy metals were prepared fresh. The concentration of stock solution of commercial-grade deltamethrin is 1.4%, which is equal to 14,000 ppm. To obtain the final concentration of interest, appropriate

volume of stock solution were added into certain volume of NA prepared. Table 2 shows the concentration of heavy metals required to meet up the concentrations of interest.

Table 2: Heavy metals used and their respective concentration in MHA

Heavy metal salts	Heavy metal cations	Stock concentration (%)	Working concentration in 100ml MHA (%)
$CuSO_4$	Copper, Cu^{2+}	1	1.5
$HgCl_2$	Mercury, Hg^{2+}	1	1.5
$ZnCl_2$	Zinc, Zn^{2+}	1	1.5

Abbreviation: $CuSO_4$=Copper (II) sulphate; $HgCl_2$=Mercury (II) chloride; $ZnCl_2$=Zinc (II) chloride