Faculty of Resource Science and Technology

ISOLATION AND CHARACTERIZATION OF BACTERIAL STRAINS THAT ARE RESISTANT TO COPPER, MERCURY AND OTHER ANTIBACTERIAL AGENTS FROM DIFFERENT SOIL ENVIRONMENTS

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DECLARATION

I hereby declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification to this or any other university or institution of higher learning.

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Jakaria Bin Tuan Haji Rambli
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List of Abbreviations

2 N HNO_3	nitric acid
Ag	argentums/silver
Aer.	*Aeromonas*
Ap	ampicillin
API	Analytical Profile Index
AP	alkaline phosphate
ara	arabinose
As	arsenic
Ca	calcium
$CaCl_2$	calcium chloride
Cd	cadmium
Cl	chloride
cm	centimetre
Cm	chloramphenicol
cnr	cobalt-nickel resistance system
Co	cobalt
CorA	cobalt resistance system of *A. eutrophus*
Cr	chromium
Cu	copper
czc	cobalt-zinc-cadmium resistance system
Cu^{2+}	Copper ion
Cu^{R}	Copper
$CuSo_4$	Copper (II) sulphate
^{o}C	degree Celsius
deo	deoxyribose
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dUTP	deoxyuridine triphosphate
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
EMB	Eosin-methylene blue
end	endonuclease 1
Ent.	*Enterobacter*
Er	erythromycin
ES	EDTA/sarcosyl
EtBr	ethidium bromide
Fe	ferum/iron
Fu	fusidic acid
g	gram
g	gravity force
G	guanine or guanosine
gal	galactose utilization
GEL	gelatinase reaction

gln	glutamine synthesis
GTG	Genetic Technology Grade
H	hydrogen
H_2S	production of hydrogen suphide gas
HCl	hydrochloric acid
Hg	mercury
Hg^{2+}	Mercury ion
$HgCl_2$	Mercury (II) chloride
Hg^R	Mercury
IND	indole production
kb	kilobase or kilobases
KH_2PO_4	potassium dihydrogen sulphate
kl.	*Klebsiella*
Km	kanamycin
lac	deletion in lactose fermentation gene
LB	Luria-Bertani medium
leu	leusine biosynthesis
LiCl	lithium chloride
M	molar
mg/ml	milligram per milliliter
mg/L	milligram per liter
MHA	Müeller-Hinton agar
MIC	Minimal inhibitory concentration
ml	milliliter
M^{2+}	divalent heavy metal cations (general representation)
mcr	methylcytosine restriction
mg	milligram
$MgSO_4$	magnesium sulphate
MIT	metal iorganic transport family
ml	millimetric
mM	millimolar
Mn	manganese
mrr	methyl-purine restriction
Mt	methicillin
MTC	maximum tolerable concentration
mtl	mannitol
mU	milli-unit
N	normal
NA	Nutrient agar
N_2	reduction of NO_2 to nitrogen gas
NaCl	sodium chloride
NaOH	sodium hydroxide
NCCLS	National Committee for Laboratory Standards
ng	nanogram
Ni	nickel
NO_2	Production of nitrogen dioxide gas

NTA	nitrilotriacetic acid
nup	nucleoside permease
OD	optical density
ODC	ornithinc decarboxylase
OH	hydroxide
ONPG	beta-galactosidase
OX	cytochrome-oxidase reaction
Pb	plumbum
Pc	Penicillin-G
PCR	Polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pmol	picomoles
Po	polonium
pro	proline
Prov.	*Providencia*
Ps.	*Pseudomonas*
^{r}	chromosome-encoded resistance phenotype
R	resistance phenotype
R.O.	reverse osmosis
rec	recombination
Rf	rifampicin
RHA	fermentation/oxidation of rhamnose
RNA	ribonucleic acid
RNaseA	Ribonuclease A
RND	resistance, nodulation and cell division transporter
rpm	revolutions per minute
rps	small ribosomal protein
^{S}	chromosome-encoded sensitive phenotype
S	sensitive phenotype
S.	*Staphylococcus*
SAC	fermentation/oxidation of sucrose
Sb	antimony
SB	suspension solution containing Tris (pH8.0) and NaCl
SDS	sodium dodecyl sulphate
Ser.	*Serratia*
Sm	streptomycin
SOB	Hanahan's medium
SOR	fermentation/oxidation of sorbitol
Sp. or spp.	Species
SS	Salmonella-Shigella medium
SSC	sodium chloride/sodium citrate (buffer)
T	thymine or thymidine
TAE	Tris/acetate/EDTA (buffer0
Taq	*Thermus aquaucus*
TBE	Tris/borate/EDTA (buffer)
Tc	tetracycline

TDA	tryptophane desaminase
TE	Tris/EDTA (buffer)
Ti	titanium
Tra	conjugative plasmid
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris-hydrochloric acid buffer
U	unit
URE	urease
UV	ultraviolet
µg/ml	microgram per milliliter
µg	microgram
UI/L	Units per Liter
µl	microlitre
µm	micrometer
µM	micromolar
V	vanadium
V	volts
VP	acetoin production
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-thiogalactopyranoside
xyl	xylose
Zn	zinc

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Isolation and Characterization of Bacterial Strains that are Resistant to Copper, Mercury and other Antibacterial Agents from Different Soil Environments

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ABSTRACT

Bacteria were isolated from soil samples taken from different locations in the Kuching industrial area. Copper- and mercury-resistant strains were selected and minimal inhibitory concentrations (MICs) for either heavy metal were determined. Mercury is more toxic than copper in both solid and liquid media. The inhibitory concentrations in solid media were higher than in liquid media. With higher MIC values, the bacterial isolated from the metal factory area were shown to be more resistant to metals compared to those from the other areas. This acts as a bioindicator of higher levels of heavy metal pollution in the metal factory area. The most dominant metal-resistant bacteria were *Pseudomonas sp.* About 50% of bacterial strains had multiple heavy metal resistance phenotypes (there were two to seven types of heavy metal). The isolates were also found to be resistant to several antibiotics, including ampicillin, kanamycin and tetracycline. The highest percentage of individual resistance detected was Cu^R, followed by CoR, Fe^R, Mg^R, Zn^R, Hg^R, Ag^R and UV^R. DNA analysis of five strains failed to show the presence of plasmids. Hence, there was no clear indication that the multiple resistances were coded by gene carried on plasmids.

Keywords: Copper, mercury, metal-resistant, bioindicator.

ABSTRAK

Bakteria telah dipencilkan daripada sampel tanah yang berasal dari lokasi industri yang berbeza di Kuching. Strain – strain yang rintang terhadap kuprum dan raksa disaring telah dipilih dan kepekatan perencat minima bagi kedua – dua jenis logam berat ditentukan. Raksa didapati lebih toksik berbanding kuprum dalam kedua – dua media pepejal dan larutan. Kepekatan perencat minima dalam media pepejal adalah lebih tinggi daripada dalam larutan. Pada kepekatan perencat minima yang lebih tinggi, bakteria yang dipencilkan didapati lebih rintang terhadap logam berat berbanding bakteria yang dipencilkan diperolehi di tempat lain. Ia berperanan sebagai penunjuk bio bagi kadar pencemaran logam berat yang tinggi di kawasan kilang besi. Strain paling dominan yang rintang terhadap logam berat terdiri daripada bakteria Pseudomonas sp. Lebih kurang 50% daripada strain bakteria ini didapati mempunyai kerintangan berganda (terdapat 2 hingga 7 jenis logam berat). Selain itu, bakteria ini juga didapati rintang terhadap pelbagai jenis antibiotik, termasuk ampisilin, kanamisin dan tetrasiklin. Peratusan kerintangan tertinggi yang didapati ialah Cu^R, diikuti oleh Co^R, Fe^R, MgR, Zn^R, Hg^R, Ag^R dan UV^R. Analisis DNA daripada lima strain gagal menunjukkan kehadiran plasmid. Maka, tiada petanda bahawa sifat kerintangan berganda dibawa oleh plasmid.

Kata kunci: Kuprum, raksa, kerintang, penunjuk bio.

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CHAPTER 1

INTRODUCTION

1.1 Background

Heavy metals are often defined as a group of metals whose atomic density is greater than 5 g/cm^3 and some of these metals play a vital role in the metabolic processes of the biota. Some other heavy metals have no biological role and are harmful to the organisms even at very low concentration (mercury). The characteristics of bacteria isolated from different environments sharing resistance to mercury (Hg^R), has been widely investigated on both biochemical and genetic level (Misra *et al.,* 1992). Although lethal to all living organisms, mercury and its compounds are non-toxic to certain bacteria, which possess resistance genes to mercurials (Osbourn *et al.*, 1997). Copper, on the other hand, is an essential trace element for many organisms as it plays a vital role in oxidation and reduction processes and occurs as a component of the prosthetic part of oxidizing enzymes. However, in excess amounts, copper is highly toxic especially to lower organisms (Jae and Cooksey, 1991). In the natural environment, especially in landfills, mercuric and copper compounds can be found in high concentrations. This is due to processes such as leaching of natural ores, weathering of rocks, and industrial pollution (Von Burg and Greenwood, 1991). The levels of mercury and copper contamination in an environment are frequently associated with the occurrence of mercury- (Hg^R) and copper- (Cu^R) resistant bacteria (Rensing and Grass 2003).

1.2 Problem statement

Kuching is a city bustling with an ever-increasing population and growing industrial activities. Extensive dumping of various industrial, agricultural, clinical, sewage, and domestic wastes at landfills, lead to release of heavy metal pollutant into the environment. Soils normally contain low background levels of heavy metals. However, in areas where agricultural, industrial areas or municipal wastes disposal sites are land-applied as fertilizer, concentrations may be much higher. Excessive levels of heavy metals can be hazardous to man, animals and plants. Most organisms especially bacteria have detoxification abilities (for example, mineralization, transformation and/or immobilization of pollutants), and they play a crucial role in biogeochemical cycles and in ecological maintenance in the biosphere (Diaz, 2004). This study describes the isolation and characterization of bacterial strains from an area that have heavy industrial activities and is contaminated with heavy metals from different soils sources. The soil samples have been taken from industrial area (Pending, Kuching). Indiscriminate releases of waste materials from industries lead to heavy metal accumulation in soil in surrounding areas. Continuous exposure of the indigenous bacterial populations there to heavy metal pollutants would eventually encourage the selection for heavy metal resistance. There is a need for detailed studies on copper and mercury resistance at the molecular level in order to understand the mechanisms of heavy metal-resistance. The objectives of this project is to screen and analyse bacterial strains that are resistant to copper and mercury that occur in the soils samples collected from the Pending riverbank at the site near to an industrial area. There are six stages of this study, starting from collection of soil samples from Pending riverbank area, isolation of bacterial strains from soils samples, screening of heavy metal that is resistant bacterial isolates, purification and

identification of bacterial isolates, analysis of minimal inhibitory concentration (MIC) and lastly is analysis of DNA by using a molecular technique.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A wide range of heavy metal resistant microorganisms had been isolated from various environments including water, soil and industrial wastes disposal sites (Hughes *et al.,* 1996). Interactions between metal and these organisms may alter the availability of metals in the general environment. The microbes, especially bacteria, can cause transformation of metal elements or compounds which often contribute towards remediation of the environment (Beveridge, 1989). Accordingly, many studies had been carried out to understand the mechanisms of heavy metal resistance in bacteria.

2.2 Heavy metal pollution

Metals usually occur naturally as ores in mineral deposits. These mineral ores have to be physico-chemically processed to yield the purer forms of the metals. Metals are introduced into the environment during mining and refining of ores, and from other sources such as the combustion of fossil fuels, industrial effluents, spraying of pesticides, and various forms of domestic wastes. Since the beginning of the industrial revolution, pollution of the environment with heavy metals, including mercury and copper are ever increasing due to its usage in agricultural activities, industrial and household chemicals. Copper is also used in electrical, plumbing, and heating equipments, and in alloys with other metals. Aluminum is introduced into the environment through its extensive use in industrial processes, in water treatment, in drugs

and food additives, and in making food containers (Sauvant *et al.,* 1999). Increasingly, organometals have been shown to enter underground water supply and the food chain, resulting in its accumulation in living organisms in the food chain, including humans (Sadhukhan *et al.,* 1997). Microbial populations in metal-polluted environments can adapt to toxic concentrations of heavy metals by developing resistance mechanism (Kasan and Baecker, 1989).

2.3 Heavy metal resistance in bacteria

The isolation and analysis of heavy metal resistant bacteria have been carried out by various groups. Over the years, heavy metal resistant bacterial strains from various genera and species have been successfully identified and characterised. Pickup *et al.* (1997) for instance, has characterised several nickel resistance isolates of *Enterobacter cloacae, Ent. Sakazalai, Ent. Agglomerans, Klebsiella oxytoca and Citrobacter freundii.* Consequently, *Staphylococcus spp, Bacillus spp, Pseudomonas spp., Streptococcus spp., Moraxella spp., E. coli, Proteus spp., Klebsiella spp.* and *Salmonella spp*. which were resistant to mercury, lead, zinc, cobalt, copper and/or chromium have been isolated by Olukoya *et al.* (1997). Additionally, copper resistance in *Citrobacter spp.* (Williams, *et al.,* 1993) and chromium (VI) resistance in some species of Pseudomonas, Aeromonas and Enterobacter (Ishibashi *et al.,* 1990; Ohtake *et al.,* 1990; Wang *et al.,* 1990; Suzuki *et al.,* 1992) have been studied and documented. Multiple resistance to certain heavy metals including silver, cadmium, lead and molybdenum, have also been reported for *Ps. aeruginosa* strains isolated from freshwater and seawater by de Vicente *et al.* (1990).

Some of these heavy metal resistant bacterial strains were shown to have metal accumulating abilities. For example, a *Citrobacter sp.*strain N14, isolated from metal-

contaminated soil (Macaskie, 1990; Macaskie *et al.,* 1992, 1995; Yong and Macaskie, 1995; Tolley *et al.,* 1995) was found to accumulate uranyl ions (Roig *et al.,* 1995; Macaskie *et al.,* 1996, 1997; Jeong *et al.*, 1997). Additionally, this isolate also exhibited the ability to accumulate radiotoxic elements such as americium and plutonium (Macaskie *et al.*, 1994, 1996). Several strains of *Thiobacillus spp.* were found to be able to accumulate arsenic, cadmium, cobalt, nickel, copper, vanadium, zinc, berilium, boron (Gomez and Bosecker, 1999; Gadd, 2000) and uranium (Mergeay, 1991), whereas a thermophilic *Archaebacteria* exhibited ability to accumulate various ores, including gold (Mergeay, 1991). Finally, metal accumulating ability has also been reported for *Alcaligenes eutrophus* by Diels *et al.* (1999).

2.4 Potential uses of heavy metal-resistant bacterial strains

Before bacterial strains or their heavy metal resistance genetic determinants can be manipulated through genetic engineering techniques for use as bioindicators and biosensors of bioavailability of heavy metals, or used in bioremediation, and bioleaching, microbial strains and their heavy metal resistance genes must be analysed. The percentage of metal-resistant bacteria in the environment can serve as an index to heavy metal pollution, which is enhanced by the presence of available metals (Baldi *et al.,* 1994). Thus, the occurrence of heavy metal-resistance bacteria can serve as bioindicators of heavy metal contamination of the environment.

2.5 Possible mechanisms of copper and mercury resistance

A cell may develop resistance by limiting metal access into cell or through alteration of cellular components. These altered functions may be mediated by various genes located on plasmids, chromosome or transposons which control metal resistance. Bacterial strains resistant to multiple metal ions have isolated and analysed by many workers. These bacteria evolved mainly due to the spread of plasmid-encoded metal resistance determinants, as in the case of *Alcaligenes eutrophas* strain CH34 (Mergeay *et al.,* 1985). In addition to the determinants, *A. eutrophus* strain CH34 harbors three *mer* determinants (resistance to Hg^{2+}) (Diels *et al.,* 1985; Dressler *et al.,* 1991) and *cop* (resistance to Cu^{2+}) determinants (Brown *et al.,* 1992). There are significant differences between chromosomal and plasmid-based metal resistance systems. Genes coding for copper and mercury resistance are usually found on plasmids instead of on chromosomes, as they are not essential for the survival of bacteria in a normal environment, without the excessive stress on their survival and neither do they code for essential component proteins of the bacteria itself.

William and Silver (1984) have shown that copper-resistant bacterial strains carry the *cop* operon that codes for the production of periplasmic copper-binding proteins. These small (30 to 50 kd) cytoplasmic proteins are given a range of names including metallothioneins, metal-binding proteins, cysteine-rich membrane-bound proteins, sequestering proteins and others. These proteins are simple products of single genes and are amplified easily to build up increased metal resistance. Bacteria can also eliminate copper that has reached the cell's interior. Essential metal resistance systems are more often chromosome-determined and more complex than plasmid-encoded resistance systems (Bruins *et al.,* 2000). Plasmid-encoded resistance systems usually involve toxic-ion efflux mechanisms. This suggests that ion efflux mechanisms

are more likely to be plasmid-borne because they can be quickly mobilized to other organisms and thus reduce the "gene-load' (Silver and Walderhaug, 1992). The *cop* operon is responsible for copper resistance in the Gram-positive bacteria *Enterococcus hirae* which contains four genes, namely *copA, copB, copZ* and *copY* (Silver and Phung, 1996). *CopA* is responsible for encoding a Cu (II) uptake ATPase, *copB* encodes a P-type efflux ATPase, while gene products of *copY* and *copZ* act as regulatory proteins that inactive the operon in the absence of Cu (II) (Silver and Phung, 1996). Periplasmic binding of Cu (II) in *Pseudomonas sp.* is coded for by this operon. Alterations in the cell wall, membrane or envelope of a microorganism are examples of metal exclusion by permeability barrier (Bruins *et al.,* 2000). This mechanism is effective in protecting essential metal-sensitive cellular components. A well studied example is the exclusion of Cu (II) by *E. coli* B as a result of structural alteration to the membrane channel protein porin (Rouch *et al.,* 1995). Extracellular binding of Cu (II) has been reported for *Kl. Aerogenes, Ps. putida* and *Arthrobacter viscosus* (Bruins *et al.,* 2000). *Mycobacterium scrofulaceum* also demonstrated intracellular accumulation of Cu (II) by sequestering it in the form of a black copper sulfate precipitate (Mergeay, 1991). On the other hand, mercury resistance is a classic example for an enzymatic detoxification system in microorganisms (Bruin *et al.,* 2000). This system allows both Gram-positive (e.g. *S. aureus* and *Bacillus sp*.) and Gram-negative bacteria (e.g. *E.coli, Ps. aeruginosa, Serratia marcescens* and *T. ferrooxidans*) to be resistant towards Hg (II) (Misra, 1992). Through this mechanism, Hg (II) was detoxified into Hg (0) (metallic mercury) which is then released to diffuse through the cell membrane and into the surrounding environment. Soils polluted with heavy metals can cause phytotoxicity and exhibit impared microbial activities. The predominance of gram-negative bacteria over gram-positive bacteria has been found in metal contaminated soils (Frostegård *et al.,* 1993). Gram-negative

bacteria for example, *Pseudomonas sp* are expected to have a higher degree of tolerance to heavy metals (Stefanowicz *et al.,* 2008).

Mercury resistance genetic factors code for the production of two inducible enzymes, namely mercuric reductase and organomercurial lyase (Silver and Misra, 1988; Misra, 1992). Organomercurial lyase catalyses the cleavage of C-Hg bonds in organomercurial compounds to liberate Hg^{2+}, which is then reduced by mercuric reductase in the presence of NADPH and sulfhydryl compounds to Hg^{o} that volatilizes out of the system due to its high vapour pressure (Schottel, 1978; Tezuka and Tonomura, 1978; Nakamura *et al.,* 1990; Misa, 1992; Pahan *et al.,* 1993). Mercury resistance is a classic example for an enzymatic detoxification system in microorganisms (Bruins *et al.,* 2000). This system allows both Gram-positive (e.g. *S. aureus* and *Bacillus sp*.) and Gram-negative (e.g. *E.coli, Ps, aeruginosa, Serratia marcescens* and *T. ferrooxidans*) to be resistant towards Hg (II) (Misra, 1992). Through this mechanisms, Hg (II) was detoxified into Hg (0) (metallic mercury) which is then released to diffuse through the cell membrane and into the surrounding environment.

Some microorganisms adapt to the presence of toxic metals by altering the sensitivity of essential cellular components, thus providing a degree of natural selection (Rouch *et al.,* 1995). This natural selection process allows protection through mutations which decrease sensitivity but does not alter the basic function of the cellular organelles or by increasing production of a particular cellular component to keep ahead of metal inactivation. The microbes may also protect themselves by producing metal-resistant components or alternate pathways in an effort to bypass sensitive components (Bruins *et al.,* 2000). Such adaptation has been found in resistance towards Cd(II) in *E. coli* (McEntee *et al.,* 1986; Mergeay, 1991).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of soil samples

Analytical grade of chemicals and reagents are used in this study. Soil samples from the industrial area (Pending, Kuching) had been collected using sterile digging tools and stored in sterile polyethylene bags. Samples are preserved at 4°C until needed.

3.1.2 Growth medium

Nutrient agar (NA) and Müeller Hinton agar (MHA) were purchased from Oxoid (UK), whereas Luria-Bertani (LB) broth was obtained from Fluka (Switzerland). All growth media were sterilized by autoclaving conditions of 121ºC for 2 hours under pressure of 15 psi.

3.1.3 Heavy metal stock solutions

The heavy metal salts used in this study are shown in Table 3.1. All chemicals were of analytical reagent grade.

Seven heavy metal stock solutions were prepared by dissolving the respective heavy metal salts in ultra-pure water. The concentration for the stock solutions were calculated based on the solubility of the heavy metal salts in soil taking into consideration the working

concentration for each heavy metal salt. The solutions were filter-sterilised through 0.22 µm membrane filter (Puradisc™ 25AS, Whatman®, USA) and stored at 4ºC. Appropriate volumes of the heavy metal stock solutions were added into MHA cooled to appropriate temperature to give desired final concentrations.

Table 3.1: Heavy metal salts that are used in this study and their respective concentrations in MHA media.

Heavy metal salts	Heavy metal cations	Stock concentrations (mg/ml)	Working concentrations (µg/ml)
$CuSO_4.5H_2O$	Copper, Cu^{2+}	200	50 – 1600
$HgCl_2$	Mercury, Hg^{2+}	15	15
$CoCl_2.6H_2O$	Cobalt, Co^{2+}	200	50 - 600
$AgNO_3$	Silver, Ag^{+}	25	15 - 25
$FeSO_4.7H_2O$	Iron (II) sulfate, Fe^{2+}	200	50 - 300
$ZnSO_4.7H_2O$	Zinc, Zn^{2+}	200	50 - 400
Mg(NO3)2.6H2O	Magnesium, Mg^{2+}	200	50 - 400

3.2 Methods

3.2.1 Isolation of bacterial strains from soil samples

Soil samples were first suspended in sterile water (1 g/ml) before being serially diluted 10-fold up to 10^{-6}. Aliquots (0.1 ml) of the 10^{-4} to 10^{-6} dilutions were spread on nutrient agar (NA) plates. Three replicates were prepared for each dilution and plates were incubated at 37ºC overnight. After incubation process, a random sample of 500 bacterial colonies from industrial