p-ISSN 0854-9028 e-ISSN 2549-9343
AGRIC
JURNAL ILMU PERTANIAN
Fakultas Pertanian dan Bisnis Universitas Kristen Satya Wacana
Jl. Diponegoro 52-60 SALATIGA 50711 - Telp. 0298-321212 ext 354
email: jurnal.agric@adm.uksw.edu, website: ejournal.uksw.edu/agric
Terakreditasi Kementrian Riset, Teknologi dan Pendidikan Tinggi berdasarkan SK No 21/E/KPT/2018

RHIIZOBACTERIA RESISTEN LOGAM BERAT ASAL TAMBANG EMAS PONGKOR INDONESIA DAN TAMBANG TEMBAGA MARINDUQUE FILIPINA

HEAVY METAL-RESISTANT RHIZOBACTERIA FROM GOLD MINE IN PONGKOR INDONESIA AND COPPER MINE IN MARINDUQUE PHILIPPINES

Erny Yuniarti[1], Ida F. Dalmacio[2], Erlinda S. Paterno[3]

[1]Indonesian Soil Research Institute, Jalan Tentara Pelajar No 12, Bogor 16111, Indonesia
[2]Department of Microbiology, University of the Philippines Los Baños
[3]Department of Soil Science, University of the Philippines Los Baños
*Corresponding author. Phone/Fax: (251) 8383745, Email: erny_yuniarti@yahoo.com

Diterima: 22 Maret 2019, disetujui 9 Juli 2019

ABSTRAK

Penelitian ini bertujuan mengisolasi, mengkarakterisasi, dan mengidentifikasi rhizobacteria dari rhizosfer tanaman yang tumbuh di situs tambang tembaga dan emas. Isolasi rhizobakter menggunakan media agar semisolid bebas N, TSA, dan SLP yang mengandung logam berat Pb, Cd, dan atau Cu. Rhizobakter hasil isolasi dikarakterisasi resistensinya terhadap Pb, Cd, Cu pada level lebih tinggi menggunakan agar SLP. Karakterisasi morfologi dan kultur yang dilakukan meliputi morfologi sel, motilitas, pewarnaan Gram, dan produksi biofilm. Identifikasi rhizobakter dengan sekuensing fragmen gen 16S RNA. Hasil penelitian menunjukkan sebagian besar rhizobakter dari situs tambang Cu (66.7% dari 21 isolat) resisten terhadap Cu (72-150 ppm) sementara sebagian besar rhizobakter dari lokasi tambang emas (77.8% dari 18 isolat) sensitif terhadap 72 ppm Cu. Sebagian besar logam Cu di dalam tanah situs tambang tembaga tidak larut dalam bentuk granul terikat batuan kerikil. Tampaknya rhizobakter dari situs tambang Cu terpapar dan beradaptasi dengan Cu yang tersedia di dalam tanah sehingga menjelaskan mengapa nilai MICnya terhadap Cu lebih rendah dari level Cu total di dalam tanah. Tiga rhizobakter resisten logam (PbSM 2.1, MGR 334, dan CuNFbM 4.1) menunjukkan kemampuan membentuk biofilm, yang diduga sebagai salah satu mekanisme resistensinya terhadap logam berat. Hasil penelitian ini juga menginformasikan bahwa tanah terkontaminasi logam berat merupakan sumber rhizobakter resisten logam yang lebih baik daripada tanah tidak terkontaminasi logam berat. Penggunaan keempat media dalam isolasi menghasilkan rhizobacteria dengan karakteristik yang lebih beragam daripada penggunaan media tunggal. Karakterisasi lebih lanjut perlu dilakukan untuk mendapatkan rhizobacteria resisten logam berat yang dapat digunakan sebagai inokulan pupuk hayati dan agen fitoremediasi.

Kata Kunci: Biofilm, kultur rhizobakteria, resistensi logam berat, tanah area tambang tembaga

ABSTRACT

The purposes of the study was to isolate, to characterize, and to identify rhizobacteria from plant rhizosphere growing in gold and copper mine. The isolation of rhizobacteria used N-free semisolid agar media, TSA, and SLP plus heavy metals (HMs), namely Pb, Cd, and or Cu. Isolated rhizobacteria were subsequently characterized for resistance to higher level of Pb, Cd, Cu in SLP media. Cultural and morphological characterization of rhizobacteria were conducted for cell morphology, motility, Gram staining, and biofilm formation. The rhizobacteria identification used sequence analysis of the 16S RNA gene fragments. The results showed that the majority of rhizobacterial from Cu mine site (66.7% of 21 isolates) were resistant to Cu (72-150 ppm) while the majority of rhizobacteria from gold mine site (77.8% of 18 isolates) were sensitive to 72 ppm Cu. Majority of Cu in the soil was insoluble as granules attaching to gravel so that rhizobacteria of Cu mine site have been exposed and adapted to available Cu. This fact, explaining that the rhizobacteria's MIC value was lower than the total Cu level in the soil. Three HMs-resistant rhizobacter (PbSM 2.1, MGR 334, and CuNFbM 4.1) formed biofilms, which was as one of the resistance mechanism to HMs. This research informed that HM contaminated-soil is better source for obtaining HM resistant rhizobacteria than HM uncontaminated-soil. The use four isolation media produce rhizobacteria which was more diverse than rhizobacteria from each isolation medium. Further characterization needs to be done to obtain HM resistant-rhizobacteria which can be used as biofertilizers and phytoremediation agent.

Keywords: Biofilm, culturable rhizobacteria, heavy metal resistance, copper mine soil

INTRODUCTION

Mining activity in many countries has brought about environmental issues such as heavy metal (HM) contamination and marginal land formation including low pH, loss of organic matter and nutrient, as well as the unstable situation of the land (Wong, 2003; Raymundo, 2006). Mine tailings are extreme environments, containing high concentrations of HMs and deficient in nitrogen and organic matter with extremely low pH of the soil. In this such area, there are some plant species tolerant to HMs and capable of growing on it (He *et al.*, 2010; Navarro-Noya *et al.*, 2012). Some native plant species growing on HM-contaminated sites were most efficient in accumulating Cu and Zn in its shoots (Yoon *et al.*, 2006). Such soil, wherein some plants tolerant to HM grow, is expected to harbor HM resistant plant growth promoting rhizobacteria which have potential for use in sustainable agriculture and phytoremediation of mine-degraded soil.

Rhizobacteria that can grow in the rhizosphere are ideal for useas bioinoculants, since the rhizosphere provides the front-line defense for roots against attack by pathogens. Thus the available selective media may isolate 1–10% of the total soil bacteria and 5–15% of the fungal population of soils (nautiyal and DasGupta, 2007). The use of dilute nutrient media particularly suited for the growth of bacteria adapted to oligotrophic conditions, and the provision of simulated natural environmental conditions for bacterial culture. This has led to the recovery of 'unculturables' from environments, likely to be due to the inclusion of essential nutrients and/or signalling molecules from the native environment (Vartoukian, Palmer & Wade, 2010). The use of various isolation medium in isolating culturable microbe are expected to cover more diverse of obtained rhizobacteria. It has been known that media such as Trypticase Soy Agar (TSA) is for isolating gram positive and negative bacteria,

N-free bromthymol medium (NFB), sucrose-minimal salts low phosphate Agar (SLP), and soil extract agar (SEA) which exclude N, reduce P concentration, and contain soil extract respectively. Furthermore, the addition heavy metal to the each medium may mimic environment condition suitable for rare rhizobacteria growth from rhizosphere of plant growing on mine tailing.

The study was intended to isolate using TSA, SLP agar, SEA, and NFB plus heavy metal, to characterize, to test the resistance of the rhizobacterial isolates to higher heavy metal concentration (Cu, Pb, and Cd), to assay biofilm production of the isolates, as well as, to identify HM metal resistant rhizobacteria from gold and Cu mined-out site. Moreover, the result of heavy metal resistance test would discuss the idea of heavy metal resistance mechanism of the isolated rhizobacteria which could be inducible or constitutive and or could be isolated from heavy meyal contaminated or uncontaminated.

MATERIALS AND METHODS

Soil and Rhizospheric Soil Sampling

Soil and rhizospheric samples of grass and hyacinth plants, as well as, talahib plants were collected from gold mine area of PT ANTAM in Pongkor, Bogor, West Java, Indonesia and Cu mined-out site in Mogpog, Marinduque, Philippines, respectively. Soil samples were submitted for chemical analysis to the Central Analytic Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH, UP Los Baños) and the Soil Analytical Chemistry Laboratory of the Indonesian Soil Research Institute (ISRI, IAARD).

Isolation of HM-Resistant Bacteria

Rhizobacteria were isolated from rhizosphere samples by serial dilution (Smalla *et al.* 2001) and spread-plate method on Solid media. Isolation media used a semisolid N-free (Nfb) media, TSA (Trypticase Soy Agar), sucrose-minimal salts low-phosphate (SLP) agar, and Soil Extract Agar (SEA). Each medium was supplemented with the following concentration of metals, which are twice a number of acceptable levels in soil as defined in the Dutch standards followed by most governments which have not developed their own formal guidelines (Chen, 2000), 72 ppm Cu ($CuCl_2$), 170 ppm Pb ($Pb(NO_3)_2$), and 1.6 ppm Cd ($Cd(NO_3)_2$) respectively, and combination of these three HMs. For inhibiting fungal growth, Nystatin (1, 0000 units/mL) and 10 mg fungicide (USP, Amresco, USA), respectively were added to media. All inoculated plates were incubated at 28°C for two to five days or until colony growth was visible.

From each dilution, 0.1 ml was transferred into a semisolid NFb in a test tube (18 x 200 mm) and incubated at 28°C until pellicle below the surface was visible. The pellicles were purified on agar plates of NFb + yeast extract (0.02 – 0.05 gL^{-1}) + NH_4Cl (0.005 M) + HM (to which the bacteria is resistant). The different colonies which grew on the plates were transferred to a new semi-solid NFb medium to examine the growth in an N-free medium. After three repetitions, cells that grew in the medium were stocked in NFb+YE+NH_4Cl slant agar.

Determination of Tolerance Levels of Isolates to Copper, Lead, and Cadmium

Nutrient broth (NB) bacterial cultures in the the log phase (12-hour-old, 6 x 10^9 cells/mL)

in amount of five (5) µL (10^5-10^6 cell mL^{-1}) of isolate were spotted into SLP agar amended with increasing concentrations of individual HM starting from 72 ppm Cu, 170 ppm Pb, and 1.6 ppm Cd, respectively. Plates were incubated for 3-5 days at 28°C.

Cultural and Morphological Characterization

Cultural characterization of biofilm formation using the microtiter plate biofilm assay (Merritt, Kadouri, and O'Toole, 2005). Each TSB culture (48-hour-old, at 28 °C) of the HM-resistant isolate was diluted to 1:100 µL with sterile dH_2O water. The bacterial suspensions (6 x 10^9 CFU/mL) were transferred into duplicate wells of a disinfected and a dried flat-bottom microtiter plate. The plate was covered and incubated at 28 °C for four days. The biofilm adhering to the bottom and wall of the washed wells were stained with 125 µL of 0.1% crystal violet for 10 min at 28 °C. The wells were washed with dH_2O and excess liquid was removed. The qualitative observation was done by visual comparison with positive control (biofilm-producing *Pseudomonas aeruginosa*) and uninoculated TSB in wells. Phenotypic characterization included their cell morphology, motility, as well as Gram stain. Test for motility was conducted by hanging drop preparation according to Aygan and Arikan (2007).

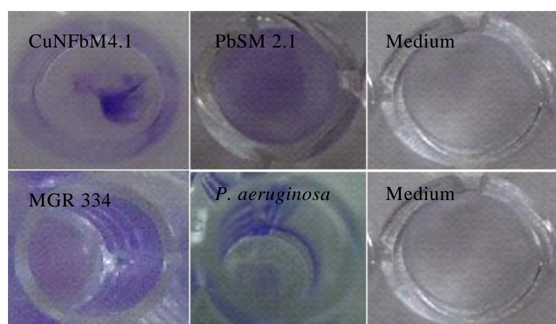

Figure 1 Biofilm formation of isolates, *Pseudomonas aeruginosa* as positive control and Medium as negative control

Morphological characterization of isolates were conducted including Gram stain, cell shape and motility.

Identification of Selected Bacterial Isolates

Harvested bacterial cells (~mid log phase, 10^9 cell/mL) were subjected to DNA kit extraction methods (Wizard Genomic DNA Purification kit, Promega) to isolate the genomic DNA of the isolates. The DNA amplification runs used oligonucleotide primers for bacterial 16S rDNA, namely 8f (AGAGTTTGATCCTGGC TCAG) and 1492r (GGTTACCTTGTTAC GACT) (Diaby *et al.*, 2007; Williams and de Los Reyes, 2006). PCR runs were performed using ESCO Micro Pte Ltd. All succeeding electrophoresis runs were done in 1% agarose gel using 3 µl aliquot of all PCR products or DNA extracts with 1 kb DNA ladder for 20 min at 100 volts and visualized under UV illumination after ethidium bromide staining. The PCR reagent mix that worked best was as follows: 8.5 mL of nuclease-free water; 1x GoTaq® Green Master Mix (Promega) containing 1.5 mM $MgCl_2$ and, 0.2 mM each dNTP; 0.4 µM of each primer; 2 µL of extracted DNA of bacterial isolates. The PCR conditions: initial denaturation and enzyme activation at 94 ºC for 5 min; 30 cycles of denaturation at 94 ºC for 1 min and 30 sec, annealing at 55 ºC for 45 sec, and extension at 72 ºC for 1 min, and a final extension at 72 ºC for 1 min. The bacterial isolates were identified by sequencing of 16S rRNA gene. Sequencing reaction was conducted in Macrogen, Inc. (South Korea). The partial 16S rDNA sequence of the isolates was analyzed by BIOEDIT program and the edited partial 16S rDNA sequences were used as a query sequence to search for a similar sequence in GenBank using Basic Local

Alignment Tool (BLAST) program (Altschul S.F., 1990). Phylogenetic trees of 16S rDNA gene sequences were generated using the neighbor-joining algorithms using the p-distance model (Saitou and Masatoshi, 1987) in Mega 6.06 software (Tamura *et al.*, 2013).

RESULTS AND DISCUSION

Soil Sample Characteristics

The analysis result of soil characteristics of gold mine in PT. Antam Pongkor Bogor and copper mine in Marinduque are presented in Table 1. Apparently, Marinduque soil classified as sandy loam soil had acid pH and was contaminated with high Cu whereas PT. Antam soil had alkaline pH and contained normal concentration heavy metal

Bacterial Isolates

Thirty-two (32) bacterial isolates were obtained from grass rhizospheric soil around the mine tailing of PT. Antam, 26 bacterial isolates from the rhizospheric soil of talahib growing on soil near the Cu mine at Marinduque. However, only 39 isolates were able to grow in further cultivation and characterization. Isolates have been coded, consecutively, the kind of HMs (Cu, Cd, Pb, and consortium of these three heavy HMs = HMC), first letter of media name (T from TSB; S from SLP), sampling location (M from copper mine in Marinduque and A from PT. ANTAM), sample number of plant (1-4 of talahib rhizosphere, 1-2 of Antam grass and 3-4 of PT. Antam hyacinth), and colony number. NFb and SE media were coded by its full name, i.e., NFb and SEM, respectively, in the name of the isolates. The number following the number of sample is colony number on media. Some isolates with MGR code originated from rhizospheric soil of talahib taken by soil brushing of the roots, according to

Table 1 Chemical and physical characteristics of gold mine from Pongkor, Bogor and Ultisol soil sample from Kentrong, Banten Province, Indonesia (0-20 cm) compared to soil from Marinduque

CHARACTERISTICS	Pongkor	MARINDUQUE
pH (1:5)		
H_2O	7.95	4.5^{++}
KCl		NT
Textural Grade (pipet)	NT	Sandy Loam
Sand (%)	NT	59
Silt (%)	NT	33
Clay (%)	NT	8
Organic matter		
C (%)	NT	0.30
N (%)	NT	0.06
C/N	-	5
Extractant (HCl 25%)		
Total P_2O_5 (mg.100 g^{-1})	NT	NT
Total K_2O (mg.100 g^{-1})	NT	0.23*****
P-Bray 1 (ppm)	NT	97*
CEC (NH4-Acetate 1N, pH 7)		
Ca ($cmol_c$ kg^{-1})	NT	NT
Mg ($cmol_c$ kg^{-1})	NT	NT
K ($cmol_c$ kg^{-1})	NT	0.23
Na ($cmol_c$ kg^{-1})	NT	NT
CEC ($cmol_c$ kg^{-1})	NT	17.00**
Pb (mg/Kg)	0.93	0.5
Cd (mg/Kg)	39.55	0.2
Cu (mg/Kg)	30.95	446.3
Hg (mg/Kg)	0.240	0.0

methods of Navaro-Noya *et al*. (2010). The isolation medium of these bacteria was NFb semisolid agar. Therefore, the isolates also stated as rhizobacteria. For these MGR isolates, naming did not follow the rule as stated above. Eight isolates, CuSEM 3.5, CuSEM 3.8, PbSEM 3.1, CdSEM 1.5, CdSEM 1.1, CdSEM 1.3, CuSEM 3.7 and CuSEM 1.3 were isolated using SEA from rhizospheric soil of plant growing in Mandrique.

Resistance of Bacterial Isolates to HM

The MIC is considered as the lowest Cu concentration at which no bacterial growth is observed (Lejon, Pascault, and Ranjard, 2010). HM resistance test was conducted on 58 isolated bacteria, however, some bacteria were difficult to cultivate and died. All the remaining isolates (39) together with their tolerance/ sensitivity to Cu, Cd and Pb are presented in Table 2. From the 21 Marinduque isolates and 18 isolates of PT. Antam, seven and 10 isolates, respectively, were sensitive to Cu only while four (CdTA 1.3, CuTA 2.2, CdTA 2.2, and CdTA 3.3) PT. Antam isolates were sensitive to all of the three HMs (Cu, Cd, and Pb). Most of the isolates revealed multiple HM resistance (double or triple), i.e., 17 isolates were resistant to Cd and Pb while another 16 were resistant to all of the three HMs. Isolates from PT. Antam, CdTA 1.1 (No. 17) and CdTA 2.1 (No. 35) were found resistant to Cu only and to Cd only, respectively.

According to Navarro-Noya *et al*. (2012), bacterial isolates growing at 1 mM (64 ppm) of Cu^{2+} is considered resistant. In general, isolates from Marinduque were more tolerant than isolates from Antam Bogor to Cu, Cd, and Pb (Tables 1). Ten isolates (CuNFb M 4.2, CdTM 2.2, PbSM 2.1, CuNFbM 4.1, CdSEM 1.5, CdSEM 1.1, MGR 335, CdTM 2.1, CdSM 2.1, and PbSM 2.2) were able to grow on medium with 125 - 150 ppm of Cu. All of the ten isolates were obtained from Marinduque around the Cu mined-out site area where the soil was severely contaminated with Cu. On the other hand, only one isolate (HMCTA 2.3) from PT. Antam was resistant to 100 ppm of Cu (Table 2).

Tabel 2 Resistance of bacterial isolates from gold ine of PT. ANTAM in Pongkor and Cu mined-out site in Mogpog, Marinduque

NO.	ISOLATE CODES	HEAVY METAL RESISTANCE			NO.	ISOLATE CODES	HEAVY METAL RESISTANCE		
1	CuSEM 3.5	Cu^{72}	$Cd^{0.6}$	Pb^{170}	21	CdTA 3.4	Cu^{-}	Cd^{15}	Pb^{260}
2	PbTA 1.1	Cu^{72}	Cd^{70}	Pb^{1040}	22	HMCTA 2.2	Cu^{-}	Cd^{20}	Pb^{260}
3	CuSEM 3.8	Cu^{100}	$Cd^{0.6}$	Pb^{1040}	23	CdTA 3.1	Cu^{-}	Cd^{20}	Pb^{290}
4	PbSEM 3.1	Cu^{100}	$Cd^{0.6}$	Pb^{1040}	24	MGR 331	Cu^{-}	Cd^{20}	Pb^{1040}
5	HMCTA 2.3	Cu^{100}	Cd^{25}	Pb^{520}	25	CuTA 2.1	Cu^{-}	Cd^{30}	Pb^{1040}
6	MGR 333	Cu^{100}	Cd^{30}	Pb^{480}	26	CdTA 2.4	Cu^{-}	Cd^{30}	Pb^{1040}
7	CuNFbM 4.2	Cu^{125}	Cd^{30}	Pb^{520}	27	CdSA 2.1	Cu^{-}	Cd^{35}	Pb^{1040}
8	CdTM 2.2	Cu^{125}	Cd^{140}	Pb^{1040}	28	CdNFbA 1.1	Cu^{-}	Cd^{35}	Pb^{1040}
9	PbSM 2.1	Cu^{125}	Cd^{140}	Pb^{1040}	29	PbTM 2.2	Cu^{-}	Cd^{70}	Pb^{1040}
10	CuNFbM 4.1	Cu^{150}	Cd^{10}	Pb^{290}	30	CdSEM 1.3	Cu^{-}	Cd^{70}	Pb^{1040}
11	CdSEM 1.5	Cu^{150}	Cd^{15}	Pb^{1040}	31	CuTM 2.1	Cu^{-}	Cd^{140}	Pb^{1040}
12	CdSEM 1.1	Cu^{150}	Cd^{20}	Pb^{1040}	32	PbTA 1.2	Cu^{-}	Cd^{140}	Pb^{1040}
13	MGR 335	Cu^{150}	Cd^{30}	Pb^{1040}	33	MGR 334	Cu^{-}	Cd^{140}	Pb^{1040}
14	CdTM 2.1	Cu^{150}	Cd^{140}	Pb^{1040}	34	MGR 5.3	Cu^{-}	Cd^{140}	Pb^{1040}
15	CdSM 2.1	Cu^{150}	Cd^{140}	Pb^{1040}	35	CdTA 2.1	Cu^{-}	Cd^{70}	Pb^{-}
16	PbSM 2.2	Cu^{150}	Cd^{140}	Pb^{1040}	36	CdTA 1.3	Cu^{-}	Cd^{-}	Pb^{-}
17	CdTA 1.1	Cu^{72}	Cd^{-}	Pb^{-}	37	CuTA 2.2	Cu^{-}	Cd^{-}	Pb^{-}
18	CdSA 4.2	Cu^{-}	$Cd^{0.6}$	Pb^{520}	38	CdTA 2.2	Cu^{-}	Cd^{-}	Pb^{-}
19	CdTA 3.2	Cu^{-}	$Cd^{0.6}$	Pb^{1040}	39	CdTA 3.3	Cu^{-}	Cd^{-}	Pb^{-}
20	MGR 2.2	Cu^{-}	$Cd^{0.6}$	Pb^{1040}					

Thirty-three (33) bacteria were able to grow on media with Cd and Pb, while only 17 were able to grow on media with Cu. Based on the MIC, HM tolerance of the bacteria from high to low was as follows: Pb >Cu >Cd. Three isolates (CdTM 2.1, CdSM 2.1 and PbSM 2.2) from Marinduque showed the highest resistance to all three HMs (150 ppm Cu, 140 ppm Cd, and 1040 ppm Pb).

The extreme characteristics of Cu mine waste-degraded sandy loam soil in Marinduque particularly high Cu HM content, low pH, low organic matter, low nutrient content in terms of nitrogen, and potassium (Table 1), as well as its location at the hilly part of the mountain, make it vulnerable to erosion. Even though efforts have been made to rehabilitate the area, greater portion still remain barren. Some plants, such as the talahib grass (*Saccharum spontaneum*) are Cu tolerant as they are able to grow in the area. Rhizosphere soil of grass and hyacinth plant yielded three bacteria (CuTM 2.1, CuTA 2.1, CuTA 2.2) which were isolated from 72 ppm Cu enriched-TSA, and four bacteria (CdTA 1.1, CdTA 1.3, CdTA 2.2, CdTA 3.3) which were isolated from 0.6 ppm Cd enriched-TSA became non Cu-resistant and non Cd-resistant in a confirmatory test on the SLP medium. Since the soil can be regarded as a community where different microorganisms live, there might be some precursors in the soil produced by microorganisms which can influence the enzyme system of the metal-resistant bacteria. Newly isolated bacteria might still have some carry-over of substances or metabolites from the soil; hence, they still maintain their resistance during the initial isolation phase, but these materials become diluted as transfer to fresh media is done.

The difference in the media used might have caused the change of the Cu or Cd-resistant isolates to become non Cu or Cd-resistant. SLP agar with low P might have provided more available HM (Cu or Cd) than TSA; hence, the MIC values of these bacteria were less than 72 ppm Cu and 0.6 ppm Cd. Low P in SLP medium is to avoid the precipitation of HM salts at 50 mg l^{-1}. Excessive P in TSA might react with the HM forming precipitation of HM salt which would not be toxic to the bacteria since most HM ions will not be able to enter the cell. Bondarezuk and Piotrowska-Seget (2013) have reported that in the *Escherichia coli,* copper ion (Cu^{+}) degrades iron-sulfur clusters of dehydratases through iron displacement, causing inactivation of the crucial enzymes. The iron released from the clusters may subsequently initiate the Fenton reaction that leads to oxidative damage inside the cell.

Also, if the resistance is plasmid-borne, it is possible that the plasmid can be lost. Some plasmids are lost quite rapidly when appropriate selective pressure is removed. Culturing bacterial isolates on agar slant without HM for short-term preservation followed by growth on NB before determination of Cu, Cd, and Pb may have caused the loss of the plasmid.

Another possibility is the community effect which could explain the above event. The isolate received resistance in the isolation medium from other members of the community where for example, one member or several members might express the resistance to HM (Cu and Cd) through biofilm production or elaborating extracellular metabolite creating untoxic form of Cu or cadmium in the isolation medium. Eventually, in this community environment, an HM-sensitive will be able to

grow near other colonies of HM-resistant bacterium and in this case, the HM sensitive bacterium can become temporarily HM-resistant.

It is noteworthy that most bacteria obtained from PT. ANTAM were Pb and Cd resistant. However, the same soil sample yielded bacteria which did not grow in the presence of 72 ppm Cu. This might imply that the Cu-resistance genes of the bacteria are inducible while lead and Cd-resistant genes are constitutive genes. Jaros³awiecka and Piotrowska-Seget (2014) and Nies (1999) explained the event by passive and unspecific resistance mechanisms where HMs bind to a variety of substrates, constitutively expressed in the cell wall and outer membrane of the bacterial cells, extracellular polymer production such as extra polysaccharides and or extra- and intracellular precipitation of Pb (II) with phosphate. The extrapolysaccharides bind Cu ions by virtue of electrostatic interaction and keep them trapped outside the cell.

Not all Cu are in available form in the soil, some Cu ores are not released or bond in gravel forms or bond to other soil components such P considering high P content in the soil. Therefore, the isolates experienced and adapted to this real available Cu which could explain that the MIC values of Cu were lower than soil Cu concentration where the bacterial isolates obtained.

Cultural, and Morphological Characteristics

Biofilm formation assay showed that isolates PbSM 2.1, MGR 334, and CuNFbM 4.1were able to form biofilm *in vitro* (Figure 1) and most of the bacterial isolates did not form biofilm. The biofilm-forming isolates originated from the rhizospheric soil of talahib grass growing in the copper mine-degraded soil in Marinduque.

Biofilms are assemblages of cells embedded in a matrix composed of exopolysaccharides, proteins, and sometimes DNA. Matrix production results in the formation of complex architectures typical of biofilms (Beauregard *et al.*, 2013). Biofilms afford a community of single or mixed species of bacteria, especially the non-spore formers, protection from the fluctuating and often severe conditions of the rhizosphere, such as desiccation, extreme pH levels, temperature, salt, and nutrient availability (Bruins, Kapil, and Oehme, 2000).

The plant growth-promoting bacterium *Bacillus subtilis* is frequently found associated with plant roots where it protects plants from infection. *B. subtilis* root attachment depends on production of an extracellular matrix that holds the cells together in multicellular communities termed biofilms. Plant polysaccharides act as an environmental cue that triggers biofilm formation by the bacterium and these plant polysaccharides can serve as carbon source used to produce the extracellular matrix. *B. subtilis* colonizes *Arabidopsis thaliana* roots and forms biofilms that depend on the same matrix genes required *in vitro*(Beauregard *et al.*, 2013). Therefore, biofilm might be a mechanism of the three bacteria on how they can be resistant to HM *in vitro* and when they are associated with roots of talahib growing on copper-contaminated soil. Other bacteria which did not form biofilm but resist to HM (in this case Cu, Pb, and Cd), might use other mechanisms to be resistant toward HM.

The biofilm that forms on roots, litter, and soil particles typically contains multiple bacterial species. There is a high prevalence of synergy

in biofilm formation in multispecies consortia isolated from a natural bacterial habitat and likewise where interspecific cooperation occurs (Ren *et al.*, 2015). This finding could explain how Cu-sensitive bacteria are able to survive on isolation media supplemented with Cu and when they are associated with roots of talahib growing on Cu-contaminated soil.

Colony observation without mentioning on what media they grew (TSA, NFB, SLP, and SEA), the morphology colonies were circular, flat, raised, smooth, mucoid, watery, white, orange, beige, yellow, bright yellow, opaque, translucent with entire margin.

Most of the bacterial cells were long or short, thick or slender rods and were Gram-negative bacilli and some isolates (10) were Gram-positive bacilli (CuSEM 3.5, CuSEM 3.8, PbSEM 3.1, HMCTA 2.3, CdSEM 1.5, CdSEM 1.1, CdTA 3.2, MGR 2.2, PbTM 2.2 and CuTA 2.2) and most of the isolates were motile (35 out of 39 isolates). The motile isolates PbSM 2.1, MGR 334, and CuNFbM 4.1 were capable of forming biofilm but other motile cells were not biofilm-forming. These results showed that motility is not associated with biofilm formation.

Identity of Bacterial Isolates

Based on 16S rDNA gene sequences, of the 18 HM-resistant isolates (11 isolates tolerant to Cu, Cd, Pb and 7 isolates tolerant to Cd and Pb) and 1 non HM (Cu, Cd, Pb)-resistant isolate (CuTA 2.2) were classified as Alphaproteobacteria (MGR 333, MGR 331), Gammaproteobacteria (PbTA 1.1, CuTM 2.1, CuTA 2.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, PbSM 2.2, MGR 5.3, MGR 335, and MGR 334), Firmicutes (CdSEM 1.1, CuSEM 3.5, CuTA 2.2, and MGR 2.2) Bacteriodetes (PbTA 1.2) and Actinobacteria (CuSEM 3.8).

From 15 isolates of Cu mined-out site in Marinduque, one of them namely CuNFbM 4.1 is most closely related to *Fulvimonas yonginensis* NR 134038 (100%). CuTM 2.1, CdTM 2.1, CdTM 2.2, PbSM 2.1, and PbSM 2.2 were found to be closely related to a member of the genus *Pseudomonas* with 100% sequence similarity. MGR 5.3 had the closest relationship with *Citrobacter amalonaticus* (99%), a member of Enterobacteriaceae. MGR 334 had closest relationship with members of Enterobacteriaceae. Thus, MGR 333 is closely related to member of the genus *Rhizobium* along with MGR 331 which clustered together with this genus (100%). Isolates CdSEM 1.1 and MGR 2.2 respectively, clustered together with the genus *Bacillus* while CuSEM 3.5 is closely related to *Lysinibacillus* sp and *Bacillus* sp. CuSEM 3.8 revealed the closest relationship (100%) with *Arthrobacter phenanthrenivorans* KC934818 (Table 2) which is known as phenanthrene-degrading bacterium (Kallimanis *et al.*, 2009). Gram stains and cell morphology characteristics of the isolates supported the taxonomic placement of the isolates which have been described above (Table 3).

The 16S rDNA sequence-analyzed isolates except CuTA 2.2 were double or triple heavy metal resistant (Cu, Cd, Pb) isolates. These bacteria covered eight different genera namely *Enterobacter*, *Sphingobacter*, *Pseudomonas*, *Rhizobium*, *Citrobacter*, *Bacillus*, *Athrobacter*, and *Fulvimonas*. The genera *Enterobacter*, *Pseudomonas*, *Rhizobium*, *Bacillus*, and *Athrobacter* are common HM-tolerant Gram-negative and Gram-positive bacteria (Ellis *et al.*, 2003; Jaros³awiecka and Piotrowska-

Table 3 Identity of selected isolates based on 16S rDNA sequences similarity analysis

NO	BACTERIA ISOLATES	MOST CLOSELY RELATED ORGANISM	ACCESSION NUM
1	Pb TA 1.1	*Enterobacter mori* S4-P4	KC851827.1
		Enterobacter cloacae CR1	AY787819.1
		Enterobacter cloacae M277	HQ651835.1
2	PbTA 1.2	Sphingobacterium multivorum DW-18	EU240955.1
3	CuTM 2.1	*Pseudomonas synxantha* A1	GQ900609.1
		Pseudomonas fluorescens MXX08012001	EU822884.1
		Pseudomonas sp.p50	EU864269.1
		Pseudomonas sp.P57	EU935094.1
		Pseudomonas azotoformans IHB B 15160	KU605225.1
		Pseudomonas libanensis IHB B 17501	KP208622.1
4	CuTA 2.1	Enterobacteriaceae	
5	CdTM 2.1	Pseudomonas fluorescens NU04	KX187323.1
		Pseudomonas proteolytica CMS64	NR 025588.1
		Pseudomonas veronii R-21933	AJ786795.1
		Pseudomonas fluorescens 20130311XB1	KC773765.1
		Pseudomonas panacis KP02	KX187317.1
		Pseudomonas sp.S-JS-8	FJ529034.1
6	CdTM 2.2	*Pseudomonas synxantha* A1	GQ900609.1
		Pseudomonas fluorescens MXX08012001	EU822884.1
		Pseudomonas sp.p50	EU864269.1
		Pseudomonas sp.P57	EU935094.1
		Pseudomonas azotoformans IHB B 15160	KU605225.1
		Pseudomonas libanensis IHB B 17501	KP208622.1
7	Pb SM 2.1	*Pseudomonas synxantha* A1	GQ900609.1
		Pseudomonas fluorescens MXX08012001	EU822884.1
		Pseudomonas sp.p50	EU864269.1
		Pseudomonas sp.P57	EU935094.1
		Pseudomonas azotoformans IHB B 15160	KU605225.1
		Pseudomonas libanensis IHB B 17501	KP208622.1
8	PbSM 2.2	*Pseudomonas synxantha* A1	GQ900609.1
		Pseudomonas fluorescens MXX08012001	EU822884.1
		Pseudomonas sp.p50	EU864269.1
		Pseudomonas sp.P57	EU935094.1
		Pseudomonas azotoformans IHB B 15160	KU605225.1
		Pseudomonas libanensis IHB B 17501	KP208622.1
9	MGR 5.3	*Citrobacter amalonaticus CECT 863*	NR 104823.1
10	MGR 335	Enterobacteriaceae	
11	MGR 334	Enterobacteriaceae	
12	MGR 333	*Rhizobium* sp.XWS-6	JQ617900.1
		Rhizobium pusense strain MB17a	KX519318.1
13	MGR 331	MGR 333	
14	CdSEM 1.1	*Bacillus* sp.BAB-4350	KM104683.1
		Bacillus bombysepticus Wang	GQ281063.1
		Bacillus thuringiensis B16	KX977387.1
		Bacillus cereus A168	GQ118339.1
15	CuSEM 3.5	*Lysinibacillus fusiformis* R110	KU752868.1
		Lysinibacillus sphaericus PRE16	EU880531.1
		Bacillus sp.JUN-3	KF228922.1
		Lysinibacillus sp.C-2-31	KT583477.1
		Bacillus cereus SEPV-3	KF228914.1
16	CuSEM 3.8	*Arthrobacter phenanthrenivorans* H31	KC934818.1
17	CuTA 2.2	*Exiguobacterium* sp.4091	JX566608.1
		Exiguobacterium indicum BR18 1A	JN644531.1
		Exiguobacterium sp.KHB11	KT368962.1
		Exiguobacterium acetylicum QD-3	FJ970034.1
18	MGR 2.2	*Bacillus* sp.13.1	KX453892.1
		Bacillus megaterium S20510	KF956591.1
		Bacillus aryabhattai IHBB	KM817252.1
		Bacillus subtilis H-3	KT273284.1
19	CuNFbM 4.1	*Fulvimonas yonginensis* 5GHs31-2	NR 134038.1

Seget, 2014; and He *et al.*, 2010). These genera also are well-known plant growth-promoting bacteria (He *et al.*, 2010 and Navarro-Noya *et al.*, 2012).

The identification result revealed that rhizobacteria isolated by four media (TSA, SLP, SEA, NFB) were more diverse than rhizobacteria isolated by each medium. Apparently, Rhizobacteria from SEA were difficult in its cultivation and preservation due to they could not have long shelf life at 4°C. The heavy metal rhizobacteria isolated by SLP (PbSM 2.1) dan NFB (CuNFBM 4.1 dan MGR 334) showed the ability to form biofilm *in vitro*. Further characterization of the successfully isolated, cultivated, and preservated rhizobacteria would give their fuctional diversity and their potential in phytoremediation and friendly sustainable agriculture.

CONCLUSION

Rhizobacteria from Marinduque were more tolerant than isolates from Antam Bogor to Cu, Cd, and Pb. Highest MIC value of Cu, Cd, and Pb were 150 ppm, 140 ppm, and 1040 ppm respectively. CdTM 2.1, CdSM 2.1, and PbSM 2.2, rhizobacteria from Marinduque exhibited highest MIC value of the three HMs.

The HM-resistant isolates from gold mine tailing in PT. ANTAM Pongkor, Bogor, Indonesia and Cu mined-out site in Marinduque, Philippines exhibited diversity in metal resistance, colony and cell morphology, as well as taxonomy where based on 16S rDNA gene sequences.

It has been requiring further characterization to select the rhizobacteria as inoculant for phytoremediation and friendly sustainable agriculture, such as the ability to accumulate heavy metal and promote palnt growth *in vitro* and in greenhouse.

ACKNOWLEDGEMENT

The authors wish to thank the Indonesian Agency for Agriculture Research and Development for funding under the project APBN. The acknowledgment is also due to the local government of Mogpog, Marinduque headed by Mayor Senen M. Livelo Jr. and the Director of PT. ANTAM, Pongkor, Bogor, West Java, Indonesia.

REFERENCES

Altschul SF., Gish W., Miller W., Myers EW., Lipman DJ. 1990. *Basic local alignment search tool*. J. Mol. Biol. 215: 403– 410.

Aygan A., Arikan B. 2007. *An overview of bacterial motility detection*. Int. J. Agri. Biol. 9:193-196.

Beauregarda PB., Chaib Y., Vlamakisa H., Losickb R., Koltera R. 2013. Bacillus subtilis biofilm induction by plant polysaccharides. http//www.pnas.org/cgi/doi/10.1073/pnas.1218984110.1621 -1630. (April 23, 2013).

Bondarczuk K. and Piotorowska-Seget Z. 2013. Molecular basis of active copper resistance mechanisms in Gram-negative bacteria. Cell Biol. Toxicol. 29:397–405.

Bruins MR., Kapil S., Oehme FW. 2000. *REVIEW Microbial resistance to metals in the environment*. Ecotoxicol. Environ. Saf. 45: 198-207.

Chen ZS. 2000. *Relationship between heavy metal concentration in soils of Taiwan and uptake by crops*. Department of

Agricultural Chemistry, National Taiwan University. P. 1-15.

Diaby N., Dold B., Pfeifer HR., Holliger C., Johnson DB., Hallberg KB. 2007. *Microbial communities in a porphyry copper tailings impoundment and their impact on the geochemical dynamics of the mine waste*. Environ. Microbiol. 9. Issue 2: 298–307.

Ellis RJ., Morgan P., Weighman AJ. Fry JC. 2003. *Cultivation-dependent and –independent approaches for determining bacterial diversity in heavy-metal-contaminated soil*. Appl. Environ. Microbiol. 69:3223-3230.

He LY., Zhang YF., HY , Su LN., Chen ZJ., Wang QY., Qian M., Sheng XF. 2010. *Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants.* Appl. Soil Ecol. 44: 49–55.

Jaroslawiecka A., Piotrowska-Seget. 2014. *Review: Lead resistance in micro-organisms*. Microbiol. 160: 12–25.

Lejon DPH., Pascault N. Ranjard L. 2010. *Differential copper impact on density, diversity and resistance of adapted culturable bacterial populations according to soil organic status*. European Journal of Soil Biology 46: 168-174.

Merritt H., Kadouri D.E., O'toole GA. 2005. Growing and analyzing Static. Current Protocols in Microbiology. John Wiley & Sons, Inc. Pp. 1B.1.1-1B.1.17.

Nautiyal CS, DasGupta SM. 2007. Screening of Plant Growth-Promoting Rhizo-bacteria. *In:* Advanced Techniques in Soil Microbiology. p.363-373

Navarro-Noya YE., Mendoza EH, Jiméneza JM., Roblero JJ., Romero EM., Rodriguez CH. 2012. *Isolation and characterization of nitrogen fixing heterotrophic bacteria from the rhizosphere of pioneer plants growing on mine tailings*. Applied Soil Ecology. 62. 52– 60.

Nies DH. 1999. *Mini-Review: Microbial heavy-metal resistance*. Appl. Microbiol .Biotechnol. 51: 730-750.

Raymundo AK., CADIZ NM., PAMPOLINA NM. and AGGANGAN NS. 2009. Initial assessment of the Mogpog abandoned mine areas for bioremediation. Unpublished report submitted to the National Academy of Science and Technology, Department of Science and Technology. *In*. Llamado AL. 2010. Bacterial Diversity Analysis of an Abandoned Mine Site in Mogpog, Marinduque Using Denaturing Gradient Gel Electrophoresis and Isolation And Identification of Potential Bioremediators. MS Thesis, University of the Philippines Los Banos.

Ren D., Madsen JS., Sorensen SJ., Burmolle M. 2015. *High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation*. Int. Soc. Microbial Ecol. 9:81–89.

Saitou N., Nei M. 1987. The neighbor-joining method: *A new method for reconstructing phylogenetic trees*. Mol. Biol. Evol. 4:406-425.

Smalla K., Wieland G., Bucher A., Zock A., Parzy J., Kaiser S., Roskot N., Heuer H., Berg G. 2001. *Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed*. Appl. Environ. Microbiol. 67(10): 4742–4751.

Tamura K., Stecher G., Eterson D., Filipski A., Kumar S. 2013. MEGA6: *Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol* Evol. 30: 2725-2729.

Vartoukian SR, Palmer RM, Wade WG. *Minireview:Strategies for culture of 'unculturable' bacteria*. FEMS Microbiol Lett. 309:1-7.

Wong MH. 2003. *Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils*. Chemosphere. 50: 775–780.

William JC., De Los Reyes III FL. 2006. *Microbial community structure of activated sludge during aerobic granulation in an annular gap bioreactor*. 54(1):139-46.

Yoon J., Cao X., Zhou Q., Ma LQ. 2006. *Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site*. Sci. Total Environ. 368: 456–464.

***