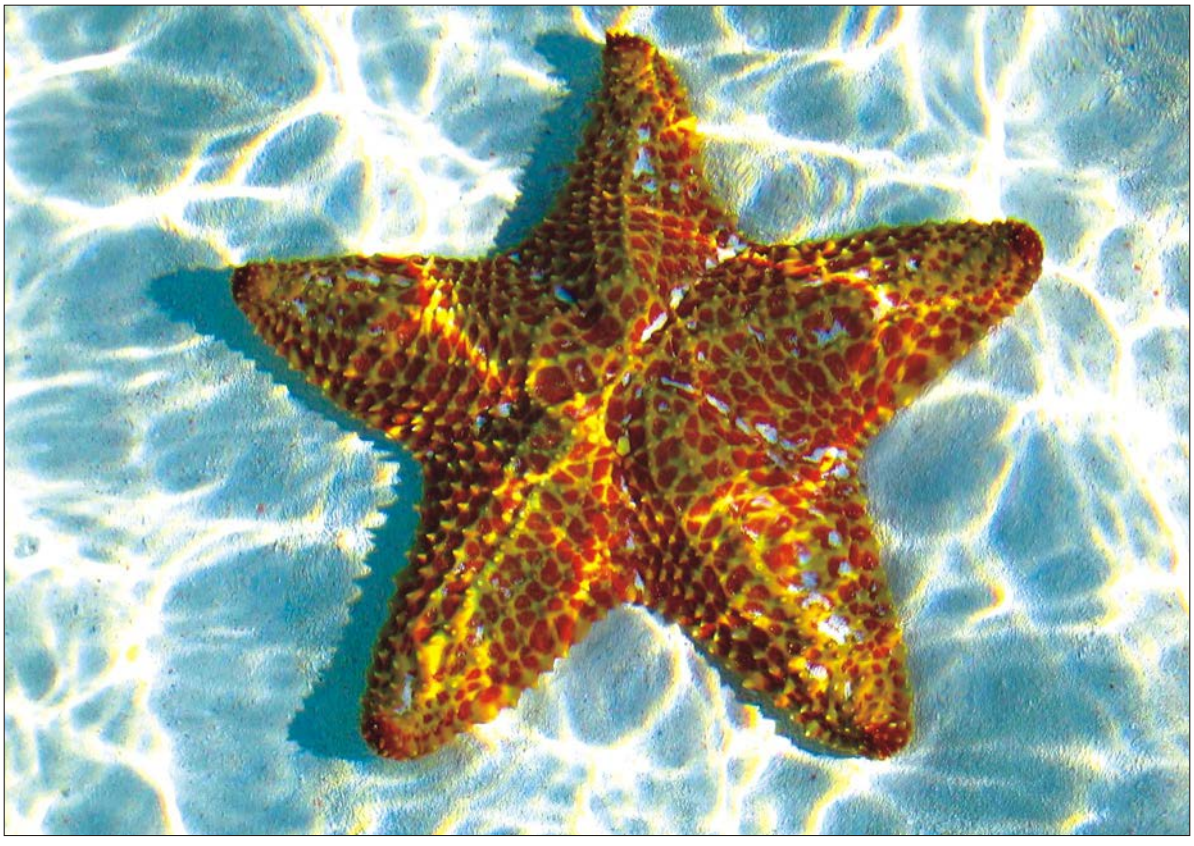

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Screening of Multi-metal Resistances in a Bacterial Population Isolated from Coral Tissues of Central Java Coastal Waters, Indonesia

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ABSTRACT

Scleractinian corals harbor diverse bacterial communities within their tissue. However, it is still not known the significant role of those bacteria in bioremediation of heavy metal contamination. The present study aimed to investigate the diversity of the bacterial community associated with the corals that have multiple resistance to heavy metals. Sixty-one coral bacteria isolated from three different life-forms of scleractinian coral samples collected from Central Java coastal waters were established by plating on Zobell's 2214E. Those isolates were screened for their resistance against Pb, Cr, Zn, at 1 mM level using agar diffusion method and 22 isolates were selected. Minimal Inhibitory Concentrations (MIC) of heavy metals were determined and different MIC of these isolates was shown to be highly resistant to Pb, Cr and Zn ions. A rapid grouping by using Repetitive Extragenic Palindromic (REP)-PCR was conducted to estimate the richness of the isolates. Three heavy metal resistant bacterial strains representing three major genetic groups were selected for further studies. Based on analysis of morphological, biochemical and 16S rDNA sequence of these isolates revealed that one strain belongs to γ-proteobacteria division while the other two belong to Firmicutes division. Isolate PL05 was closely related to *Pseudoalteromonas* sp. while PL12 and PL22 isolates were closely related to *Virgibacillus* sp. This work provides the first evidence of bacteria possessing multiple resistances against heavy metals can be recovered from corals.

Key words: Bacteria, coral, heavy metal, resistant, *Virgibacillus* sp., *Pseudoalteromonas* sp., Java Sea

INTRODUCTION

Coral reef is one among tropical coastal ecosystems of the world beside mangrove and seagrass (Kathiresan and Alikunhi, 2011). Coral reef ecosystems have a high species diversity that contain hundreds of reef fish, corals, plants and animals (Cesar *et al*., 2003; Veron, 1986). Due to the abundance of unique chemical properties from certain coral types, coral reefs have been viewed as the medicine cabinets of the sea (Tacio, 2004). Corals have shown a great potential for finding effective chemical agents that provide a large proportion of bioactive compounds with different biological activities (Radjasa and Sabdono, 2009; Shahbudin *et al*., 2011). Because of its potentials, Bruckner (2002) suggested that coral reefs could be the major source of drugs for the next decade.

Corals are host of bacterial life that living on seawater around corals, the surface and tissue of corals and their interactions have been studied in detail (Rohwer *et al.*, 2001, 2002; Lampert *et al.*, 2006; Koren and Rosenberg, 2006; Ibrahim, 2008; Lins-de-Barros *et al.*, 2010). However, little is known about the functional role of bacteria associated with scleractinian corals (Rohwer *et al.*, 2002). Even the understanding of their functional role in coral reef ecosystems is still ignored, Ritchie (2006) reported that coral bacteria play significant role in the antibiotic activity and pigment production. In addition, some investigators stated that coral bacteria were also play important role in cycling of nitrogen, carbon, sulphur and phosphate (Siboni *et al.*, 2008; Sharon and Rosenberg, 2008; Raina *et al.*, 2009). Furthermore, several studies reported that coral bacteria play functional role in phosphatase enzyme activity (Al-Shehri, 2006), anti-pathogenic bacterium *Streptococcus equi* (Radjasa *et al.*, 2007) and health and disease of corals (Friaz-Lopez *et al.*, 2002). Sabdono and Radjasa (2008) reported that coral bacteria are capable of degrading organophosphate pesticides. Coral bacteria resistant to heavy metal can be used in remediation efforts of various environments, since they can grow under variable salinity and temperature (Koren and Rosenberg, 2006).

Central Java is the third most-populous province in Indonesia and one of the fastest growing provinces in commercial and industrial sectors. Wastes from residential areas, rivers, industries and agricultures have intensified and through migration, runoff and infiltration, they make a way to the coastal waters. Several studies concerning the marine pollutants of Java coastal waters reported the impact of anthropogenic activities on heavy metal contamination (Booij *et al.*, 2001; Takarina *et al.*, 2004; Sabdono, 2009). Because of their toxicity, persistence and bioaccumulation problem, heavy metals are one of the most serious polluting agents in marine environments (Blackmore, 1998; Selvin *et al.*, 2009). Studies showed that marine pollution had severely impacted the microbial ecology (Danovaro *et al.*, 2003; Dell'Anno *et al.*, 2003; Boyd, 2010).

Heavy metals such as lead, chrome and zinc were emphasized in this study due to their extensive use in wood preservation, electroplating, metal-finishing and chemical industries of Central Java. These heavy metals were also detected at high concentration in dead coral tissues (Sabdono, 2009). Heavy metals become toxic to the cell when present at concentration above trace amounts (Nies, 1999). The objective of this study was to investigate the diversity of the bacterial community found within the coral tissues that have multiple resistance to metals.

MATERIALS AND METHODS

Sampling and coral bacterial isolation: Corals representing 3 different life forms (branching, massive, sub-massive) were collected from Central Java coastal waters in July 2008. Specimens of the corals *Porites lutea, Galaxea fascicularis* and *Pocillopora damicornis* were collected randomly by scuba diving at depths of 2 to 5 m. Individual specimens were placed separately in plastic bags to avoid contact with air and brought to the surface. The individual samples in the plastic bags containing natural seawater were processed within a few hours after collection. Tissue samples were removed from the skeleton with a sterilized scrapper and the exposed surface tissues were removed with a sterile scalpel blade. The resultant tissues were serially diluted spread on a half-strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al.*, 2000).

Screening of metal resistant isolates: A total of 61 bacterial strains isolated from three coral species (*P. lutea*, *G. fascicularis* and *P. damicornis*) were screened for their resistance to three heavy metals according to the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). Filter paper disks, 8 mm in diameter (Toyobo, Co, Japan), were soaked in solutions of the appropriate heavy-metal salt (lead, chrome or zinc, supplied as $Pb(NO_3)_2$, $K_2Cr_2O_7$ or $ZnCl_2$) at 1 mM concentrations. The disks were then placed on the surface of plates that previously inoculated with 0.1 mL of isolates. Each plate contained one disk lacking the heavy metal salt and three disks containing each concentration of heavy metal salt. The plates were then incubated at 28°C for approximately 72 h. At the end of incubation period, the zones of inhibition were measured as indicator for resistance. Zone measurements were recorded as the distance from the edge of the zone to the edge of the disk. Isolates that had a zone size (clearance zone) less than 1.00 mm were considered as resistant strain (Rani *et al.*, 2010). Isolates showing resistant to 1 mM metals were further tested at higher concentrations.

Minimum Inhibitory Concentration (MIC) of the heavy metal resistant coral bacteria was determined by gradually increasing 0.5 mM of the heavy metal concentration. The starting concentration used was 1.5 mM. MIC was noted when the isolates formed zone inhibition greater than 1.0 mm in size.

Microscopic and biochemical characterizations: Selected bacterial strains highly resistant to heavy metals were grown in Zobell 2216E medium and underwent further microscopic and biochemical evaluations. Photomicrograph was used to determine the morphology of the isolates. While standard gram staining, motility and biochemical characterizations based on Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) were used to determine their biochemical properties.

Rep-PCR amplification and grouping of isolates: To determine genetic relatedness among bacterial isolates, DNA fingerprinting were performed according to the method of Sabdono and Radjasa (2008). In the rep-PCR, BOX AIR primer (5'-CTACGGCAAGGCGACGCTG ACG-3'; Versalovic *et al.*, 1994) was used. Genetic grouping analyses of selected isolates was carried out by making matrixes from the positions of bands on the gel which were then analyzed using Free Tree program (Pavlicek *et al.*, 1999). The Tree View ver.1.6.6 program was used in constructing the tree (Page, 1996).

DNA extraction, PCR amplification and sequencing of 16S rDNA: DNA extraction, PCR amplification of partial 16S rDNA of bacterial strain and purification of PCR products were also carried out based on the method of Sabdono and Radjasa (2008). Primers [(forward primer 8-27: 5'-AGAGTTTGATCCTGGCTCAG-3' (Weisburg *et al.*, 1991) and reverse primer 1510-1492: 5'-GGTTACCTTGTTACGACTT-3' (Reysenbach *et al.*, 1992) were used to amplify 16S rDNA.

Sequencing and phylogenetic analysis: Based on phylogenetic grouping, the isolates each representing major genetic group were selected and used for DNA sequencing. The sequencing and phylogenetic analysis were conducted according to the method of Sabdono and Radjasa (2008). The PCR product was purified and concentrated with Microcon-100 microconcentrators (Amicon, Beverly, MA, USA) according to manufacturer's instructions. Sequencing was carried out with a SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) and

an automated sequencer (the ALF DNA sequences: Pharmacia LKB Biotech, Uppsala, Sweden). The nucleotides sequences obtained from partial sequencing of 16S rDNA were then compared for homology to the BLAST database. A phylogenetic tree was constructed using maximum-likelihood analysis. Clustal X software was used for multiple alignment/pairwise the DNA sequence (Thompson *et al.*, 1997). Phylogenetic analysis was performed with the Phylogenetic Analysis Using Parsimony (PAUP Ver.4) software package (Swofford, 1998). Bootstrap analysis of 1,000 replicates was performed to estimate robustness of the tree.

Nucleotide sequence accession numbers: Nucleotide sequences of the 16S rDNA from three heavy metal bacterial resistant strain obtained in this study have been deposited in the GeneBank database under accession number HQ659245 to HQ659247. The accession numbers of 16S rDNA of other strain cited and used as comparison in this study.

RESULTS AND DISCUSSION

Isolation and screening of coral bacteria resistant to heavy metals: Initial screening using 1 mM metal concentration indicated that out of 61 coral bacterial isolates, 10 isolates (16.4%) were susceptible to any heavy metal tested while 22 (36.1%), 13 (21.3%) and 16 (26.2%) isolates were resistance to three, two and one metal(s), respectively (Table 1). When the three different life forms of corals were compared, the highest percentage of metal resistant bacteria (77.3%) was originated from coral species *P. lutea*. While, the percentage of metal resistant bacteria isolated from *G. fascicularis* and *P. damicornis* were 11.1 and 14.3%, respectively. No literature evidences could be compared to heavy metal resistant bacteria isolated from scleractinian corals. However, there were many studies regarding bacteria associated with other marine invertebrates that resistant to heavy metals. Jeanthon and Prieur (1990) reported that heterotrophic bacteria isolated from two deep-sea hydrothermal vent polychaete Annelids were resistant to high concentration of metal. Most of those isolates (92.3%) displayed multiple resistance to cadmium, zinc, arsenate and silver and tolerated high amounts of copper. Selvin *et al.* (2009) found out the heavy metal resistance pattern of the bacteria associated with a marine sponge *Fasciospongia cavernosa*. Similar studies on heavy metal resistance by marine actinomycetes isolated from saltpan soil have been reported (Deepika and Kannabiran, 2010). In addition, heavy metal resistant bacteria were isolated from sediment in the Uppanar Estuary, South East Coast of India (Karthikeyan *et al.*, 2007) and Sunchon Bay, South Korea (Kamala-Kannan and Lee, 2008). The highest percentage metal resistant bacteria found in coral *P. lutea* indicated that this coral species could be used as material source of bacterial isolation. Al-Rousan *et al.* (2007) reported that *Porites* corals (massive) have a high tendency to accumulate heavy metals. Since the bacteria associated with coral *Porites* were continuously subjected to high levels of metals, this condition could create the emergence of metal

Table 1: Comparison of the no. isolates of multiple metal resistant bacteria of the three different corals

		No. of isolates resistant to			
Coral species	Total No. of isolates	3 metals	2 metals	1 metal	0 metal
P. lutea	22	17	4	1	0
G. fascicularis	18	2	7	6	3
P. damicornis	21	3	2	9	7
Total	61	22	13	16	10

resistant bacteria. The 22 isolates showing resistance to 3 metals were reassessed further for higher concentrations.

MIC (Minimum Inhibitory Concentration) of 22 isolates: The resistance levels of the 22 coral bacterial isolates to three metal toxicities and their range varied (Table 2). The strains evaluated had a wide resistant range to lead toxicity from 2.0 to 10 mM. They had a narrower range to chromium toxicity from 2.5 to 5 mM and an extremely narrow resistant range to zinc toxicity of <1.5 up to 2.5 mM. Nies, 1999 stated that the bacteria are able to tolerate beyond Cd 0.5 mM, Zn 1.0 mM, Cu 1.0 mM, Pb 5.0 mM and Ni 1.0 mM could be considered as extreme. By using this definition, the results of this study demonstrated that 6, 3 and 2 isolates were extremely resistant to lead, chromium and zinc, respectively. While, the remaining had high and moderate level of resistance. Compared to the previous study, both the highest MIC of lead and chrome in this study were slightly higher than that of *Frankia* strain (Richards *et al.*, 2002). However, those MIC value was lower for lead and similar for chrome to that of reported by Nieto *et al.* (1989). The resistance of coral bacteria to heavy metals could be induced due to the industrial and domestic wastewater from coastal regions of Central Java. High concentrations of heavy metals in the coral tissues were found in this polluted coral reef region (Sabdono, 2009). Since bacteria living in the coral tissue were continuously subjected to heavy metal toxicity, this condition could stimulate the emergence of metal resistant coral bacteria. The differences of these results are due to the levels of metal pollution and type of organic structures (Gillan *et al.*, 2005; Bezverbnaya *et al.*, 2005).

Table 2: The MICs of the heavy metals tested against coral bacteria determined by zone of inhibition

	MIC (mM) of		
Isolate	Pb	Cr	Zn
PL01	2.5	2.5	<1.5
PL02	5.0	2.5	<1.5
PL03	2.5	2.5	<1.5
PL04	5.0	2.5	<1.5
PL05	10.0	2.5	2.5
PL07	5.0	2.5	<1.5
PL08	5.0	2.5	<1.5
PL09	5.0	2.5	<1.5
PL10	5.0	2.5	<1.5
PL12	10.0	2.5	<1.5
PL15	10.0	2.5	<1.5
PL16	5.0	2.5	<1.5
PL17	5.0	2.5	<1.5
PL18	5.0	2.5	<1.5
PL20	10.0	5.0	<1.5
PL21	10.0	5.0	<1.5
PL22	10.0	5.0	2.5
GF01	2.0	2.5	<1.5
GF15	2.0	2.5	<1.5
PD04	2.5	2.5	<1.5
PD10	2.5	2.5	<1.5
PD16	2.0	2.5	<1.5

Characterization of selected bacterial isolates: Based on the repetitive-PCR results and constructed dendrogram of the isolates, three groups were created at which similarity level (Fig. 1). The 16S rDNA from strain PL22, PL05 and PL12 representing each of the three groups were characterized and sequenced to obtain information on their identity. Microbiological characteristics and the results of DNA sequencing of those isolates are presented in Table 3 and 4. The two selected isolates (PL22 and PL12) are gram positive while PL05 isolate is gram negative. All three selected isolates were rod-shaped, aerobe, motile, endospore former with positive catalase and oxidase activity. All of them did not produce any kinds of pigments and have no ability to metabolite all the 6 sugars tested. Different from PL05 isolate, the isolate of PL22 and PL12 grew on 10% NaCl concentration.

Analysis of 16S rDNA sequences revealed the presence of two major groups of bacteria: (1) Firmicutes and (2) γ-proteobacteria. BLAST analysis of PL22 and PL12 isolates revealed that these strains were close relative, with 99% similarity, of *Virgibacillus marismortui* and *Virgibacillus* sp. DV2-60, respectively. While, BLAST analysis of PL05 isolate revealed that this isolate is a close relative, with 99% similarity, of the strain *Pseudoalteromonas* sp. B281 (Table 4). The 16S rDNA sequences of these bacteria were submitted to GenBank (Accession no. HQ659245 to HQ659247). Several heavy metal-resistant bacteria isolated from marine environments have been identified. Stuart *et al.* (2009) reported coastal marine bacteria *Synechococcus* sp. tolerance to copper. Bacteria *Pseudomonas* sp. and *Delftia* sp. resistant to metals isolated from sea water and sediment of Persian Gulf were reported by Zolgharnein *et al.* (2010). De Souza *et al.* (2006) found psychotropic bacteria resistant to heavy metal and antibiotic isolated from Antarctic marine water. In addition, Selvin *et al.* (2009) reported bacteria *Streptomyces* sp., *Salinobacter* sp., *Roseobacter* sp., *Pseudomonas* sp., *Vibrio* sp., *Micromonospora* sp., *Saccharomonospora* sp. and *Alteromonas* sp. resistance against heavy metals isolated from marine sponge.

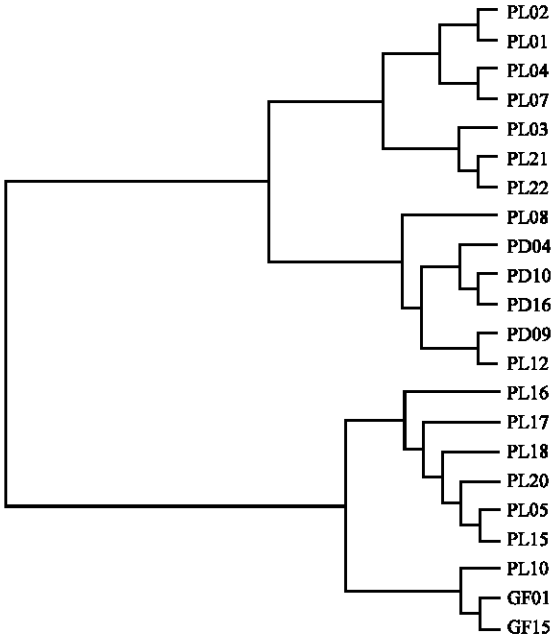

Fig. 1: Dendrogram of multi metal resistant isolates (PL22, PL12, PL05 were further selected for DNA sequencing)

Table 3: Microbiological characterization of three selected isolates

Characters	PL22	PL12	PL05
Gram	+	+	-
Form	rod	rod	rod
Acid fast	-	-	-
Spore	+	+	-
Motility	+	+	+
Aerobic	+	+	+
Anaerobic	-	+[F]	-
Catalase	+	+	+
Oxidase	-	+	+
Glucose acid	+	+	+
Carbohydrate (OF)	NC	NC	NC
Growth with 10% NaCl	+	+	-
Nitrate reduced	+	+	-
Gas from glucose	-	-	-
Indol	-	-	-
ONPG	-	-	-
VP	-	-	-
Hydrolysis of			
Starch	-	-	-
Urea	-	-	-
Casein	+	-	+
Acid from AAS medium			
L-arabinose	-	-	-
Salicin	-	-	-
Sucrose	-	-	-
Xylose	-	-	-
Cellobiose	-	-	+
Galactose	-	-	-
Rafinose	-	-	-
Gelatinase	v	v	-
Growth on 50°	-	-	-
Pigment production	-	-	-
Utilization of nitrate	-	-	-

Sign+: Positive result; sign -: Negative result; NC: No change; v: No assayed; F: Facultative

Table 4: Characterization of representative heavy metal-resistant coral bacteria

Strain	Group	Closest relative	Acc. No.	Homology (%)	No. isolate[†]
PL22	Firmicutes	*Virgibacillus marismortui*	AB305203	99	7
PL12	Firmicutes	*Virgibacillus* sp. DV2-60	GQ407264	99	6
PL05	γ-proteobacteria	*Pseudoalteromonas* sp. *B281*	FN295784	99	9

[†]No. of metal resistant isolates represented by the strain based on BOX-PCR fingerprint data

To estimate genetic affiliation of the heavy metal-resistant isolates among coral-associated bacteria, a neighbor-joining tree including identified isolates and representative marine microorganisms is constructed. A phylogenetic analysis of the 16S rRNA data for selected strains belonging to the group of the Firmicutes and Proteobacteria produced the dendrogram shown in Fig. 2. This comparison was made to determine the species to which the three selected isolates are most closely related and how closely the three taxa are related to each other. The PL12 and PL22

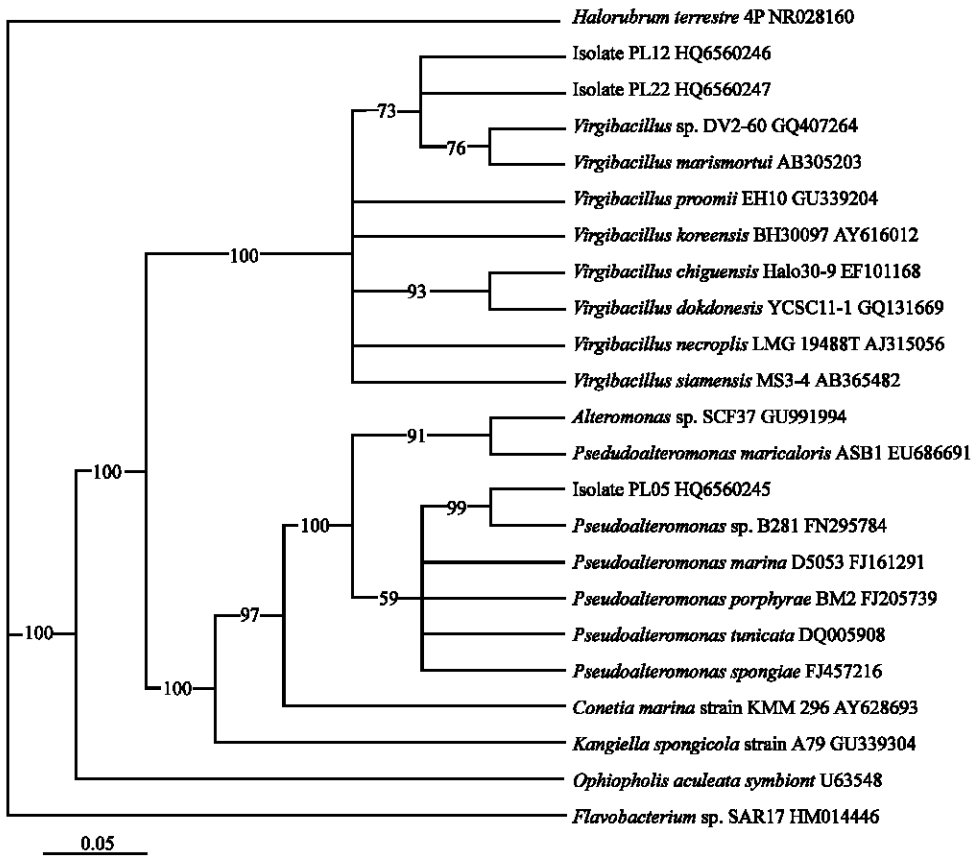

Fig. 2: Phylogenetic tree based on the 16S ribosomal DNA sequence data showing the relationships of representative strains with the most closely related bacteria identified in the GenBank database. *Halorubrum terrestre* was used as out group. Bar indicated 5% dissimilarity of sequences

isolates were in the same cluster of phylogenetic tree *Virgibacillus* sp. while the PL05 isolate was in the cluster of *Pseudoalteromonas* sp. Many researchers have reported the structure of coral-associated bacterial communities isolated from different coral species. Bourne and Munn (2005) reported that the majority of microbial community obtained from coral tissue *P. damicornis* was γ-proteobacteria, whereas the coral mucus was dominated by α-proteobacteria. While Koren and Rosenberg (2006) revealed that a large diversity of bacteria associated with coral tissue of *Oculina patagonica* were *Pseudomonas* sp., α-proteobacteria and *Vibrio* species. The results of the present study showed that the bacterial diversity associated with corals was different from the previous reported. The differences in bacterial community structure could be explained as an effect of heavy metal pollution on the coral reef ecosystem. Webster *et al.* (2001) reported that the total density and counts of microbial communities associated with the sponge *Rhopaloeides odorabile* were significantly reduced in response to Cu^{2+} concentrations. Ager *et al.* (2010) stated that anthropogenic pollution will reduce bacterial species richness, loss of species and change in bacterial community structure. In addition, coastal pollution has an impact on the microbial communities inhabiting healthy coral tissues (Klaus *et al.*, 2007).

It is interesting to note that the PL22, PL12 and PL05 isolates showed high multiple resistant against Pb, Cr, Zn heavy metals. This raises the possibility the use of these bacteria as potential candidates for remediation efforts of heavy metal contaminated coral reef ecosystems. The genus *Virgibacillus* constitutes a diverse group of gram-positive bacteria, rod-shaped and spore forming (Peng *et al.*, 2009). This members of genus are ubiquitous in different marine environments reflect their wide functional properties. Gupta *et al.* (2008) reported that *Virgibacillus* sp. produce extracellular thermostable serine alkaline protease while Kuhlmann *et al.* (2008) reported that this genus could also produce ectoine as a microbial osmoprotectant. In addition, the genus *Virgibacillus* sp produce salt-activated extracellular proteinases (Sinsuwan *et al.*, 2007) and possess inhibitory activity against fouling bacteria (Kanagasabhapathy *et al.*, 2005). The member of genus *Pseudoalteromonas* is a rod-shaped, motile, gram-negative bacteria that usually found in association with marine eukaryotic hosts such as sponges and algae (Bowman, 2007). This genus is well known to produce inhibitory compounds against surface competitors (Thomas *et al.*, 2008). Moreover, Hedlund and Staley (2006) reported that genus *Pseudoalteromonas* could degrade polycyclic aromatic hydrocarbons while Mimura *et al.* (2008) reported that this genus could absorb trybutylin.

CONCLUSION

Bacteria living in the coral tissue are complex and diverse. Further studies are needed to investigate their mechanisms of metal resistance that could be useful in the bioremediation of contaminated coral reef ecosystems. This paper reported that coral bacteria *Virgibacillus marismortui* PL22, *Virgibacillus* sp. PL12 and *Pseudoalteromonas* sp. PL05 showed high multiple resistant activity against Pb, Cr, Zn heavy metals.

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