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Original Paper

CADMIUM REMOVAL BY A BIOREDUCPIUN CORAL BACTERIUM *Pseudoalteromonas* sp. STRAIN CD15 ISOLATED FROM THE TISSUE OF CORAL *Goniastrea aspera,* JEPARA WATERS

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ABSTRACT

Seventeen strains of bacterial symbionts which are resistant to heavy metals, were isolated from the tissue of coral Goniastrea aspera, from Awur Bay, Jepara Waters. Screening procedures involving solid and liquid synthetic media containing Cd^{2+}, resulted in the selection of seven Cd resistant strains, which showed a quantitative removal of Cd^{2+} by range of 68-90% of the initial Cd^{2+} concentrations (5 ppm) tested. One of these strains, CD15, was selected further to examine its molecular and physiological characteristics. Based on 16S ribosomal DNA sequencing and microbial characterization, the CG15 isolate is closely related to Pseudoalteromonas sp. This is the first report on the natural Cd metal tolerance levels of coral bacteria

Key words: Cadmium, *Pseudoalteromonas*, coral tissue, Jepara waters

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INTRODUCTION

Human activities have had a major impact in degradation of coral reefs since the 1970s (Pandolfi *et.al.*, 2003). Coral reefs are experiencing stress and bleaching periods from a variety of natural and anthropogenic sources, such as global warming, sedimentation and pollution. Heavy metals are well known marine pollutants of Java coastal waters that come from such sources as industrial discharges, urban/agricultural run-off, sewage treatment discharges and anti-fouling paints (Susuki *et al.*, 1992; Booij *et al*., 2001; Takarina *et al*., 2004).

It is well understood that corals harbor diverse microbial communities. Their surface is covered by muco-polysaccharides, which provides a matrix for bacterial colonization leading to the formation of biofilm-forming microbial communities (Kim, 1994). This bacteria associated with corals may have a major role in the fate of these contaminants, since bacteria may volatilize or precipitate metals and transform them into toxic organic derivatives (Ford and Ryan, 1995; Ehrlich, 1997).

Metals are required by the cell in trace amounts for biochemical reactions, however, at higher concentrations, they can have toxic effects (Nies, 1999; Burnley, 2000). The cell may use the low concentrations of some heavy metals in redox reactions to stabilize electrostatic forces, enzymes and DNA binding

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proteins, and to counteract negatively charged cellular building blocks and metabolites (Nies, 1999).

The toxic effects of Cadmium on microorganisms are well known and derived from several mechanisms (Silver 1996; Bowman *et al.*, 1990; Wang, *et al*., 1997; Nies 1999). Disruption of protein function can occur through binding of Cadmium to sulphydryl groups (Cunningham and Lundie, 1993; Jungmann, *et al.*, 1993). Moreover, binding of Cadmium to nucleotides leads to single-strand breaks in cellular DNA that result in prolonged lag phase, decreased growth rate, lower cell density, or death for bacteria at levels below 1 ppm of Cadmium (Wang, *et al*., 1997). Mason and Jenkins, (1995) stated that Cadmium can affect the function of metal requiring enzymes and proteins, and cause oxidative stress by generating hydroxyl radicals .

Recently, research in heavy metal removal from wastewaters and sediments has emphasized on the development of materials with increased affinity, capacity, and selectivity for target metals (Gadd and White, 1993; Tortura, 1996). The use of microorganisms to sequester, precipitate, or alter the oxidation state of various heavy metals has been extensively studied (Shen and Wang, 1993). Some workers have studied the mechanism of heavy metal biosorption using pure microbial species (Nakajima and Sakaguchi, 1986). As a results, a wide variety of bacteria have been reported to oxidize heavy metals in fresh and marine waters, soils, and sediments (Erlichs, 1996), including *Alcaligenes eutrophus* (Nies, 1995), *Gluconobacter oxydans* (Robakis *et al.*, 1985), *Staphyococcus aureus* and *Staphylococcus lugdunensis* (Diels *et al*., 1985). However, to date there are no reports concerning bacterial natural resistance to heavy metals isolated from corals.

The purpose of this study were to isolate, select and identify indigenous bacteria associated with coral capable of removing Cd metal. It is expected that still quite a few parts of unexplored Cd resistant culturable coral-associated bacteria exists in the reef environments. Such information may be desirable, as some of these bacteria may serve beneficial purposes as the source of indigenous Cd heavy metal removal for coral conservation related to the use of bacteria as bioremediator .

MATERIAL AND METHODS

Sampling and coral bacterial isolation

Colony of *G. aspera* was found on open reef bottoms and form morphologically similar colonies of individual, upright tubes. Specimens of the hard coral *G. aspera* were collected by scuba diving at depths of 3 to 5 m. Individual specimens were placed separately into plastic bags to avoid contact with air and brought to the surface. The samples were kept individually in plastic bags containing natural seawater until processing 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 ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al*. 2000).

Heavy metal resistance test

The disk method designed by Burnley (2000), was used in this experiment. In this method, filter paper disks, 8 mm in diameter (Toyobo,

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Co, Japan) were soaked in solutions of Cadmium heavy-metal salt at 1 mM concentrations or soaked in dH2O as a negative control. The disks were then allowed to dry and subsequently sterilized. Once sterile, the disks were placed on the surface of 5% glycerol plates on which was spread 0.1ml of the appropriate isolates at an Optical Density 620nm (OD620) of 0.8-1.0. Each plate contained one disk lacking the Cd heavy metal salt and one disk containing each concentration of heavy metal salt. These plates were incubated at 28^0C for approximately 72 h, at which time the zones of inhibition were measured. Zone measurements were recorded as the distance from the edge of the zone to the edge of the disk. A zone size greater than 1mm was scored as sensitive.

Cadmium removal

Isolates were grown in 50-ml shake flasks containing 20 ml of Cadmium-supplemented medium (5 ppm Cd) inoculated with 0.5 ml of cells (1% inoculum). Flasks were incubated at 30°C and agitated at 200 rpm in a shaker bath. Optical density at 600 nm (OD600) and pH were monitored. Selected samples were transferred to Eppendorf tubes and centrifuged for 3 min at 12,000 x g. The supernatant was drawn and stored at 20°C for Cadmium analysis. The Cadmium concentration in the supernatant was determined with a Perkin-Elmer 2380 atomic absorption spectrometer at 228.8 nm with a cadmium lamp.

Microscopic and biochemical characterizations

All cells used in microscopic characterization were grown in Zobell 2216E medium. The morphologies of isolate were determined from photomicrograph. Gram staining, motility and biochemical characterizations were determined based on *Bergey's Manual of Determinative Bacteriology*.

DNA extraction, PCR amplification and sequencing of 16S rRNA gene fragments

DNA extraction, PCR amplification of partial 16S rRNA gene of bacterial strain, purification of PCR product and subsequent sequencing analysis was performed according to the method of Radjasa et al. (2007). The determined DNA sequence of strain was then compared for homology to the BLAST database.

Phylogenetic analysis.

A phylogenetic tree was constructed using maximum-likelihood analysis. Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA. CLUSTAL X was used for multiple alignment/pairwise the DNA sequence (Thompson et al., 1997). Phylogenetic analysis was performed with the PAUP*4.0 (Phylogenetic Analysis Using Parsimony) software package (Swofford, 1998).

RESULTS AND DISCUSSION

Divalent cations are essential nutrients for bacteria and are required as trace elements at lower concentrations. However, at higher concentrations, these ions are potentially toxic. Seventeen strains of bacterial symbionts were isolated and tested for Cd metal resistance by agar diffusion methods (**Table 1 and Fig.1**). There were widespread among coral bacteria on Cd resistance. Six isolates, namely CD02, CD10, CD11, CD14, CD15 and CD16,

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showed no inhibition zonal formations and were selected for further study. Whereas, the other eleven isolates formed zone of inhibition, with diameters range from 1.59 to17.67 mm. According to Burnley (2000), these eleven bacteria were sensitive to Cd metal since the size of zone of inhibition were greater than that of 1 mm.

The difference of Cd metal resistance among coral bacteria was mainly due to their interference with microbial metabolism or their altering of the physicochemical environment of cells. Microorganisms have developed several mechanisms at the cellular and molecular levels to overcome such changes in their external environment (Nies, 1992).

It was conceivable that the disk-sensitivity method was not adequate to determine resistance or sensitivity of Gram-positive bacteria to Cadmium and other heavy-metal ions. Perhaps penetration of Cadmium ions into the medium surrounding the disk is too slow to adequately show resistance or sensitivity to cadmium by growing bacteria. Since there should be no diffusion problems using heavy-metal salts dissolved in liquid media, the six bacterial selected were determined their resistance or sensitivity by examining the growth response to Cadmium in broth cultures.

Table 1. Response of *Pseudoalteromonas* strain CD15 to $CdCl_2$ concentration determined by zone of inhibition

ISOLATE	Inhibition Zone (mm)			
	1	2	3	Mean
GN01	3.50	3.90	3.70	3.70
CD02	-	-	-	-
CD03	3.90	2.20	2.20	2.77
CD04	5.72	4.30	4.30	4.77
CD05	9.80	9.80	9.40	9.67
CD06	3.50	2.78	2.68	2.99
CD07	1.88	3.00	2.24	2.37
CD08	5.18	4.30	5.50	4.99
CD09	1.96	2.48	0.34	1.59
CD 10	-	-	-	-
CD 11	-	-	-	-
CD 12	7.02	7.00	17.50	7.84
CD 13	6.42	8.50	14.40	7.11
CD 14	-	-	-	-
CD 15	-	-	-	-
CD 16	-	-	-	-
CD 17	3.00	3.00	11.220	11.07

- : no inhibition zone formed

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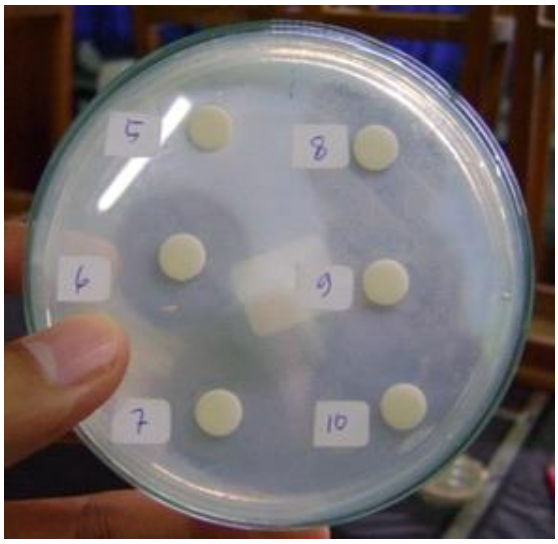

Fig.1. Agar diffusion test of coral bacteria against Cd

The six bacteria selected demonstrated their capability of removing Cd metal in broth cultures ranged from 68 to 90.4 % (**Table 2**). Bacterial indigenous to heavy metal-containing environments have developed several distinct mechanisms of heavy metal tolerance (Wang *et al.*, 1997).

Table 2. Cadmium removal by coral bacteria

Isolate	Adsorption (%)
CD02	70,6
CD10	69,8
CD11	68,0
CD14	77,8
CD15	90,4
CD16	68,2

A common plasmid-encoded mechanism employs heavy metal efflux pumps, which specifically capture and eject undesirable metals through the cell membrane (Nies, 1992), or sequestered by adsorption to the cell wall (Mullen *et al*.,1989; Scott and Palmer, 1990) or by binding to detoxifying ligands, proteins, or polymers (Aiking *et al*., 1984; Nies, 1992; Wilkfors *et al*., 1991). In addition, Aiking *et al*. (1984) stated that microbially mediated precipitation of heavy metals as insoluble sulfides, carbonates, phosphates, or hydroxides can also reduce the bioavailable concentration of the toxic ions. Microbial enzymes can convert metal ions into organometallic compounds, thereby detoxifying or volatilizing the metal ions (Nies, 1992). CD15 isolate showed the highest Cd removal, was selected for polyphasic study.

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Table 3. Microbiological characterization of CD15 isolate

Assay	Result:
Gram	+
Form	Rod
Acid Fast	-
Spora	-
Length cell > 3 µm	-
Motility	+
Aerobic	+
Anaerobic	-
Catalase	+
Oxydase	+
Glucose acid	+
Carbohidrate (OF)	NC
Growth with 10% NaCl	-
Nitrate reduce	-
Gas from glucose	-
Indole	-
ONPG	-
VP	-
Hydrolysis of:	
- **Starch**	-
- **Urea**	-
- **Casein**	+
Acid from AAS medium :	
- **L – Arabinose**	-
- **Salicin**	-
- **Sucrose**	-
- **Xylose**	-
- **Celibiose**	+
- **Galactose**	-
- **Rafinose**	-
Gelatinase	-
Growth on 50^0	-
Pigment production	-
Utilization of Citrat	-

+ : reaction; - = no reaction; nc: no change

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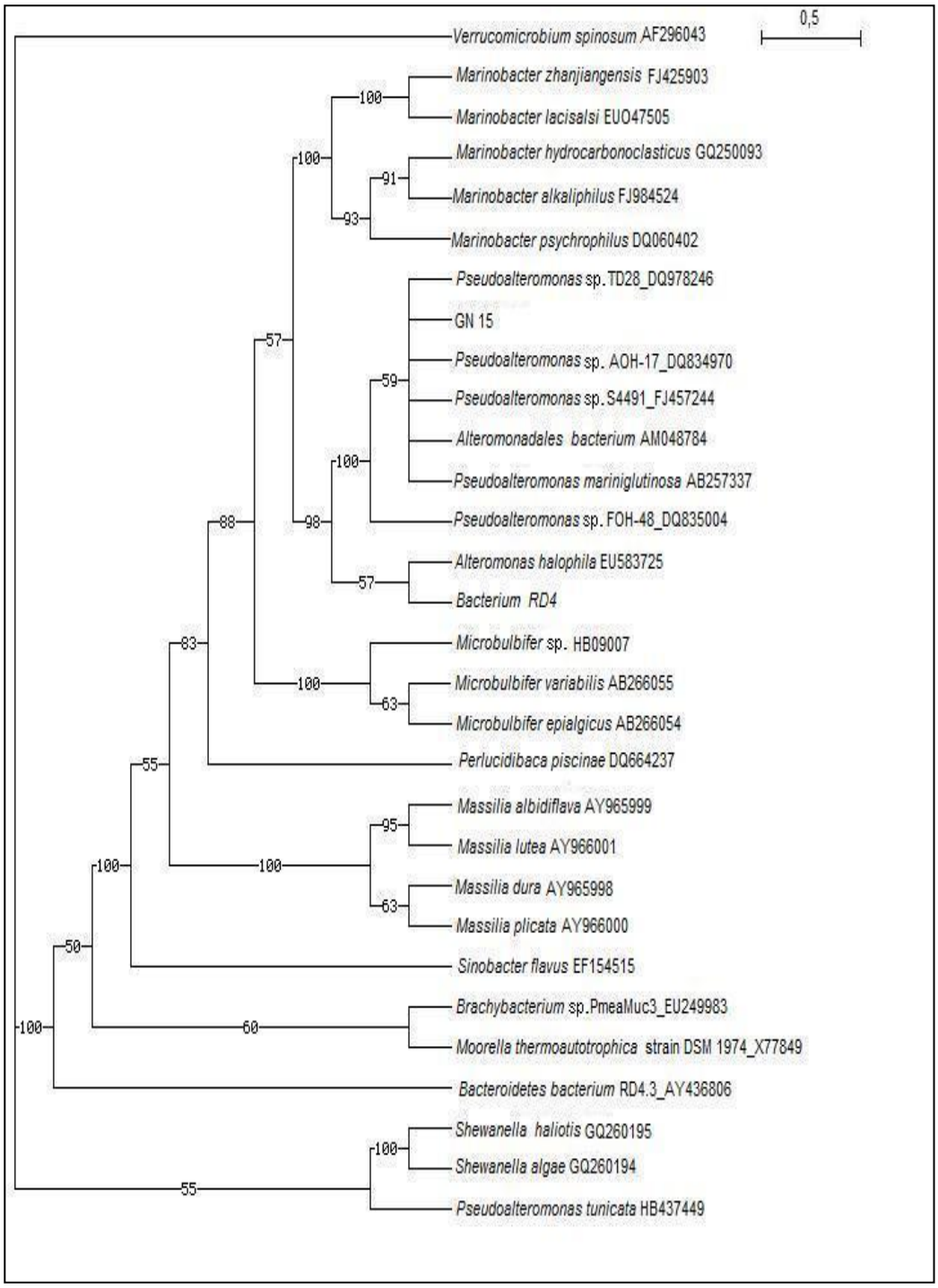

Fig.2. Phylogenetic tree based on comparative 16S rRNA gene sequence analysis of strain CD15. *Verrucomcrobium spinosum* was used as *outgroup.*

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The results of the morphological and biochemical identification experiments are shown in the Table 3. CD15 bacterial isolate are rod shaped, no spore former. The response to the biochemical reactions tested during the study showed that CD15 isolate has positive reaction to catalase, glucose and xylose. This bacterium can hydrolyze urea, but not for starch and casein. Based on the molecular analysis data, a phylogenetic tree was constructed by comparing nucleotide sequences with available 16S rRNA sequences. The CD15 bacterial isolate was identified as *Pseudoalteromonas* sp. Constructed phylogenic tree was presented in the **Fig. 2.** *Pseudoalteromonas* sp. was grouped within the genus Pseudoalteromonas.

This bacterial genera represent the common marine bacteria and have been reported as oxytetracycline-resistant (Dang *et al.,* 2007), tributyltin by tributyltin resistant (Mimura *et al*., 2008), mercury-resistant (Iohara *et al*., 2001), paralytic shellfish toxins degrading (Donovan, 2009) and chitin degrading (Techkarnjanaruk and Goodman, 1999).

CONCLUSION

The results of this investigation indicate that the potential use of coral bacteria to remove Cd metal in contaminated marine sites. Characterizing the pathway for adsorption, identifying the genes and enzymes involved in this process represent areas for further investigation.

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