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ISOLATION AND CHARACTERIZATION OF MERCURY RESISTANT MARINE BACTERIA FROM THE COASTAL AREA OF CHENNAI, INDIA

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Abstract: *Among all the heavy metals, Mercury (Hg) has no known essential biological function and is extremely toxic. Despite the fact that estuarine sediments are often severely polluted with mercury, only a limited number of studies have focused on the mechanisms of adaptation in marine sediment microbial communities to mercury contamination. Metal-resistant microorganisms may be useful as indicators of potential toxicity to other forms of life and are important in studies of mechanisms, determinants and genetic transfer of microbial metal-resistance. The primary objective of this study was to isolate and enumerate mercury-resistant bacteria from various locations in the coastal regions of Chennai. Seventy marine bacteria were isolated from Sea water and sediments collected from three different locations. Bacteria highly resistant to mercury (BHRM) isolated from seawater and sediment samples were tested for their growth in the presence of different concentrations of mercury and other heavy metals. It has been found that 5 marine bacteria were highly resistant to all the heavy metals tested. These BHRM were characterized morphologically and biochemically. These strains have been subjected to plasmid curing and the same has confirmed that these strains are having mercury resistance which may be plasmid mediated. The hypothesis put forth for this study that bacterial strains capable of Hg resistance can also tolerate, detoxify or biotransform a variety of other toxicants.*

Keywords: *mercury resistance, plasmids, marine bacteria, sea water, biotransformation*

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INTRODUCTION

Heavy metals are elements that are characterized by relatively high density and high relative atomic weight. Usually their atomic number is greater than 20 (Shen et al., 2002; Chibuike and Obiora, 2014). Although heavy metals are naturally present in the soil, both natural (geologic processes such as volcanic eruption) and anthropogenic activities increase the concentration of these elements in higher quantities that pose serious threat to plants and animals (Chibuike and Obiora, 2014). Some heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, V, and Zn are required in minute quantities by organisms. However, excessive amounts of these elements can become harmful to organisms. Other heavy metals such as Pb, Cd, Hg, and As do not have any beneficial effect on organisms and are thus regarded as the "main threats" since they are very harmful to both plants and animals (Chibuike and Obiora, 2014). We are just beginning to understand the metabolism of heavy metals and to use their metabolic functions in biotechnology, although heavy metals comprise the major part of the elements in the periodic table (Nies, 199). Bacteria have been examined that have developed very efficient and different mechanisms for tolerating heavy metals. Often normal toxic levels of metals have no effect on cell growth of resistant strains. In many organisms, the genes controlling metal resistance are carried on plasmids, which provide the bacteria with a competitive advantage over other organisms when metals are present. Bacteria have been examined that have developed very efficient and different mechanisms for tolerating heavy metals (Trevors et al., 1985).

During the last two decades, extensive attention has been paid on management of environmental pollution and its control due to hazardous materials such heavy metals (Umrania, 2006). Remediation of heavy metal contaminated sites both in aquatic and terrestrial realms has been a challenge for a long time. Bioremediation is the use of organisms (microorganisms and/or plants) for the treatment of polluted soils. It is a widely accepted method of soil remediation because it is method of remediation which utilizes few natural processes. Heavy metals cannot be completely degraded during bioremediation but can only be transformed from one organic complex or oxidation state to another and this is termed as biotransformation (Chibuike and Obiora, 2014). Microbial bioremediation serves as an alternative and effective strategy to remove toxic contaminants from a polluted environment. It could be achieved through the interaction of microbes with the toxic

contaminants, which leads to immobilization, compartmentalization, and concentration of pollutants rather than their degradation and elimination from the environment (Pushpanathan et al., 2014). Phytoremediation is the application of plant-associated microbes to remove/contain pollutants from the air, soil and water. This technology has opened up promising areas of research in the field of phytoremediation technology and is rapidly expanding and also commercialized (Rajkumar et al., 2012).

There have been several studies which utilized microorganisms for heavy metal bioremediation. Heavy metal bioremediation by a multi-metal resistant endophytic bacteria L14 (EB L14) isolated from the cadmium hyperaccumulator *Solanum nigrum* L. was characterized for its potential application in metal treatment (Guo et al., 2010). For the last several decades, metal resistant microorganisms including marine bacteria have been considered a potential alternative for heavy metal recovery and bioremediation resulting in the development and refinement of many bioremediation technologies for removal of toxic metals form contaminated environments.Interestingly, these bioremediation technologies are economically viable, environmental friendly and value added processes (Naik et al., 2012). The advantage of using marine bacteria for bioremediation in situ is the direct use of organisms in any adverse conditions without any genetic manipulation (Dash et al., 2013). So, studies were concentrated on marine bacteria for their applications in heavy metal bioremediation. *Bacillus thuringiensis* PW-05 was isolated from the Odisha coast and was found to resist 50 ppm of Hg as HgCl2 as well as higher concentrations of CdCl2, ZnSO4, PbNO3 and Na2HAsO4 (Dash et al., 2010). Hence, in the present study, an attempt has been made to isolate marine bacteria from the sediment and water samples collected from the coastal area of Chennai, India. A total of 70p marine bacteria were isolated from which 5 efficient marine bacteria found to have exceptional mercury resistance. The study also report the mercury resistance may be due to resistant plasmids.

MATERIALS AND METHODS

Experimental Conditions

All the experiments were carried out at room temperature (35°C) unless otherwise stated. All the bacterial strains used in the study were stored in sterile filtered sea water in eppendorf tubes at refrigerated conditions (4°C).

Sample Collection

Sea water and inter-tidal zone sediments were collected at low tides from the Kanathur, Kalpakkam and Tiger cave coastal area along Chennai, India. The coastal regions of Chennai receive a wide variety of industrial, urban and shipping related effluents and are among the most pollution affected zones along the Indian coast. Sediments were extruded into sterile tubes and either processed immediately or stored on ice and processed within 18–24 h. Samples used were 10 ml of sea water in 90 ml of sterile distilled water [master dilution 10^{-1}] and 10 g of sediments in 95 ml water [master dilution10^{-1}]. The samples were mixed well and kept for 15 min at shaking conditions in 150 rpm.

Isolation of marine bacteria

The samples were serially diluted in distilled water blanks up to 10^{-6} dilutions. One ml each of diluted samples was taken from each dilutions and pour plate technique was performed on Nutrient Agar Prepared in aged sea water. The plates were incubated at 35°C for 2 days and the diverse and distinct colonies observed. Morphologically distinct bacterial colonies were sub cultured in Nutrient agar and their colony morphology were observed. Isolated colonies were preserved as sea water stocks at 4°C.

Screening the marine bacteria for mercury resistance

All the isolated organisms were screened with mercury in different concentrations like 10 µM, 100 µM, 250 µM, 500 µM, 1 mM and 10 mM. Bacteria were spot inoculated on heavy metal amended medium (HMAM), nutrient agar in plates. Resistance of strains against the mercury in the various concentrations was examined after an incubation period of 2 days at room temperature. The Media used were normal nutrient agar medium without any heavy metals as control and HMAM prepared with Nutrient agar and desired concentration of mercury or other heavy metals. The bacteria that grow well in mercury amended plates equivalent to that of control plates were noted as Mercury Resistant Bacteria (MRB) and were subjected to further characterization studies. Their growth was characterized in a +++ scale system where + means poor growth, ++ means average growth and +++ means good growth when compared to control plates. Here – means no growth.

Spectrum of tolerance to various heavy metals

The selected MRB were further characterized for their ability to tolerate other heavy metals such as nickel, copper, chromium and iron at three different concentrations 10 µM, 100 µM

and 250 µM. Non metal amended nutrient agar plates were considered as controls. In test plates nutrient agar medium was amended with different concentrations of the heavy metals tested. The bacteria that grow well in HMAM plates equivalent to that of control plates were noted as resistant ones. Their growth was characterized in a +++ scale system where + means poor growth, ++ means average growth and +++ means good growth when compared to control plates. Here – means no growth.

Biochemcial characterization of MRB

Basic staining and biochemical characterization tests were done as described by Cappuccino and Sherman (2004) and the results were interpreted with the key provided in the Bergy's Manual of Determinative Bacteriology (Holt et al., 2004).

Plasmid curing with ethidium bromide

Plasmid curing is an experiment to completely remove any plasmids in a bacterial cell. The logic behind using this experiment in our study is to find whether the plasmid less bacteria could able to grow in mercury amended medium. If the bacterium grows in HMAM even after plasmid curing, the mercury resistance may not be plasmid borne. If the bacteria could not grow in HMAM after plasmid curing it shall be concluded that the bacterium lost its heavy metal resistance after plasmid curing thus confirms the resistance is plasmid borne.

One ml of culture from the 24 h old broth culture was centrifuged at 8000 rpm for10 minutes and the supernatant was discarded. To the pellet, ethidium bromide 50µg + 200µl sterile water mixture was added and incubated for 2 h. After 2 h of incubation, samples were serially diluted up to 10^{-4} and spread plated performed in HMAM. (Mercury 250 µM). In control (without ethidium treatment) samples were serially diluted up to 10^{-8} and spread plated on HMAM. The number of colonies appeared on HMAM and control plates were counted as per the treatment.

Plasmid curing with acridine orange

The isolates that exhibit highest level of metal resistance were subjected to plasmid curing experiment to confirm the plasmid mediated metal resistance. Acridine orange (10mg/10ml) was used as the plasmid curing agent. Nutrient broth was prepared and amended with acridine orange to reach a final concentration of 400µg/ml and sterilized. A similar set of NB without acridine orange was also prepared and sterilized. They were labeled as acridine amended (AO^+) and non acridine amended (AO^-). After autoclaving and

cooling, 150µl from each of the strains were transferred to these tubes. The tubes were incubated in the environmental shaker at 35^0C under 175 rpm for 2 h after which it was serially diluted and spread plated on NA plates. The number of colonies appeared on HMAM and control plates were counted as per the treatment (Jayaprakashvel et al., 2014a).

RESULTS AND DISCUSSION

Mercury and its compounds are distributed widely across the earth. Many of the chemical forms of mercury are toxic to all living organisms. However, bacteria have evolved mechanisms of resistance to several of these different chemical forms, and play a major role in the global cycling of mercury in the natural environment. (Osborn et al., 1997). It has been hypothesized that bacteria resistant to high concentrations of mercury would have potential capacities to tolerate or possibly degrade a variety of toxic materials and thus would be important in environmental pollution bioremediation (De et al., 2003). Coastal environments are reporedt to have high populations of mercury resistant bacteria (MRB). Ramaiah and De (2003) have reported for the first time the occurrence of aerobic heterotrophic bacterial isolates capable of growth with 250 µM Hg. Such MRB grew with higher concentrations of many other toxic xenobiotics than the Hg sensitive ones. Based on the unusually high populations of viable MRB they have proposed that that many marine bacterial species are selected, possibly through acquisition of plasmids and/or transposable elements and modifying Hg, whose concentration, according to recent studies, is on the rise in marine habitats.

Hence, in the present study attempts were made to isolate MRB from marine samples of coastal area around Chennai, India. These bacteria were then also characterized for resistance to other heavy metals and the possible role of plasmids in the heavymetal resistance of selected strains was also notice. The bacterial load in samples collected at different places varies with samples and locations. Seventy morphologically distinct colonies were isolated from serially diluted samples and were sub-cultured to purity on Nutrient agar medium prepared in sea water (Table 1). Similar to our study, other researchers have also isolated mercury resistant bacteria from seawater. The marine bacteria strains S1, S2 and S3, were isolated on seawater culturing medium containing Hg(2+), Cd(2+), Cr(6+) or Ni(2+) at concentrations of 20 mg L(-1) and more. The isolates showed tolerance to these heavy metals. S1 grew in the presence of 120 mg L(-1) of Hg(2+) and accumulated Hg(2+) at pH 4-

10 (Deng and Wang, 2012). The special organomercurial-volatilizing bacteria found in the seawater and sediments of Minamata Bay were screened to develop a method for the removal of mercury to produce clean products. A total of 104 mercury-resistant bacteria that could grow on an agar plate containing 40 µg/ml of HgCl2 were isolated from Minamata Bay (Nakamura et al., 2001). Marine bacteria have been isolated frequently by our research group for various reasons such as bioprospecting and environmental applications. Our studies have also reported many *Pseudmonas* spp from marine environment (Jayaprakashvel et al., 2014 a,b,c; Bhagat et al., 2014; Vinothini et al., 2014; Vijayan et al., 2012)

In the present study, out of 70 strains isolated from coastal sediments and seawater, only 5 strains showed resistance to mercury in the concentrations upto 250 µM. None of the strains could grow beyond that concentration of mercury. The selected MRB are KLSW6225, KLSW6226, KLSW6228, KLSW6229 and KLSW6230 (Table 2). Incidentally, all these strains were isolated from the seawater samples collected at Kalpakkam, Chennai. These five strains were also screened for their resistance towards other metals like nickel, copper, chromium and iron in different concentrations up to 250 µM. It has been found that all the five strains have showed remarkable tolerance to all the four heavy metals that too in all the tested concentrations (Table 3). Thus the hypothesis of De at al. (2003) i.e. bacteria resistant to high concentrations of mercury would have potential capacities to tolerate or possibly degrade a variety of toxic materials has been confirmed and thus these five selected MRB strains from marine environment can be potentially used in environmental pollution bioremediation

In the present study, attempts were made to broadly classify the selected MRB from marine environment. So, few biochemical and staining techniques were done. By and large, culturally, all these five bacterial strains were looking very similar. All these five strains are gram negative and actively motile rod shaped bacteria. All of them are oxidase and catalase positive indicating that they are aerobic or facultatively anaerobic. showed rod shape in simple staining. All the five selected MRB strains were growing well in Kings B medium and all of them have exhibited yellow green fluorescence when observed under UV illumination in the KB Agar plates. This has indicated that they could be members of the genus *Pseudomonas.* To conform further these straisn were streaked in Cetrimide Agar where

good growth has been observed. Hence based on the growth, biochemical and staining experiments these strains are confirmed to be members of the genus *Pseudomonas*.

Several members of the genus *Pseudomonas* were reported by others. A mercury-resistant bacterial strain *Pseudomonas putida* Spi3, was isolated from polluted river sediments and characterized to reduce ionic mercury to metallic mercury. The strain was used to remediate in laboratory columns mercury-containing wastewater produced during electrolytic production of chlorine (von Canstein et al., 1999). The *Pseudomonas putida* strain SP1 was isolated from marine environment and was found to be resistant to 280 µM $HgCl_2$. SP1 was also highly resistant to other metals, including $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, and $ZnSO_4$, and the antibiotics ampicillin (Ap), kanamycin (Kn), chloramphenicol (Cm), and tetracycline (Tc) (Zhang et al., 2012). Hence it can be concluded that the present study was also succesuful in isolating five mercury resistant marine bacterial strains and all of them are found to be members of the genus *Pseudomonas*.

Some bacteria are able to resist heavy metal contamination through chemical transformation by reduction, oxidation, methylation and demethylation. One of the best understood biological systems for detoxifying organometallic or inorganic compounds involves the mer operon. The mer determinants, in these bacteria are often located in plasmids or transposons and can also be found in chromosomes (Nascimento and Chartone-Souza, 2003). Plasmid curing is one of the useful experiments to confirm the plasmid mediated special characteristics of bacteria (Jayaprakashvel et al., 2014a). Here in this case, if the plasmid cured organisms failed to grow in antibiotic amended medium it implies that the organisms are having the plasmid mediated resistance. Plasmid curing with ethidium bromide treatment was performed with these five MRB strains with 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} dilutions. Numbers of colonies in all isolates were counted. All had colonies in the range of too numerous to count indicating that the experiment was unsuccessful. Hence other plasmid curing methods are to be adopted. Similalry, *Pseudomonas* sp isolated from the Bay of Bengal (Madras coast) contained a single large plasmid (pMR1) of 146 kb. Plasmid curing was not successful with mitomycin C, sodium dodecyl sulfate, acridine orange, nalidixic acid or heat (Rajini Rani and Mahadevan, 1992). Then, plasmid curing experiment was taken further with another curing agent acridine organge. The five isolates that exhibited highest level of metal resistance were subjected to plasmid curing experiment with Acridine orange

to confirm the plasmid mediated metal resistance.The plasmid cured strains are further sub cultured in cetrimide and mercury amended media to detect their preference in growth. Both in terms of population and growth, cetrimide agar has supported the good population and growth of MRB when compared to HMAM (Table 4). Through the present study, it can be suggested that a slightly higher incidence of plasmids occurred in bacteria isolated from marine sediments compared to that of the marine water. The findings suggested that plasmids are highly ubiquitous and predominant in most heavy metal resistant bacteria. It was also observed that the frequencies of plasmids in the heavy metal resistant bacteria are higher than that of the natural bacteria.

CONCLUSION

Thus the study concludes that seawater in industrially polluted coastal environments habrour more numbers of mercury resistant bacteria. The bacteria that are having mercury resistance also exhibit resistance to other toxic heavy metals. Further, it has been found that the mercury resistance of selected five mercury resistant marine Pseudmonas spp. may be plasmid borne.

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Table 1: sample collection and isolation of marine bacteria

Sl. No.	Sampling location	GPS Details (from Google Maps)	Sample	No. of Samples collected	No. of bacteria isolated
1	Kanathur	12.850287, 80.248604	Sea water and Sediment	2	18
2	Tiger Caves, Mahabalipuram	12.655253, 80.210050	Sea water and Sediment	2	37
3	Kalpakkam	12.526287, 80.165843	Sea water and Sediment	4	15
Total				**8**	**70**

Table 2: Screening of Marine Bacteria for mercury resistance

Sl.No	Isolate Code No	Growth of marine bacteria at different concentrations of mercury					
		10mM	1mM	500 µM	250 µM	100 µM	10 µM
1	KLSW 6225	-	-	-	+++	+++	+++
2	KLSW 6226	-	-	-	+++	+++	+++
3	KLSW 6228	-	-	-	+++	+++	+++
4	KLSW 6229	-	-	-	+++	+++	+++
5	KLSW 6230	-	-	-	+++	+++	+++

Table 3: Spectrum of heavy metal resistance exhibited by mercury resistant marine bacteria

Sl. No	Metals Screened	Isolate Code No	Growth of MRB in the presence of other heavy metals of different concentrations		
			250 µM	100 µM	10 µM
1	Nickel	KLSW 6225	+++	+++	+++
		KLSW 6226	+++	+++	+++
		KLSW 6228	+++	+++	+++
		KLSW 6229	+++	+++	+++
		KLSW 6230	+++	+++	+++
2	Iron	KLSW 6225	+++	+++	+++
		KLSW 6226	+++	+++	+++
		KLSW 6228	+++	+++	+++
		KLSW 6229	+++	+++	+++
		KLSW 6230	+++	+++	+++
3	Copper	KLSW 6225	+++	+++	+++
		KLSW 6226	+++	+++	+++
		KLSW 6228	+++	+++	+++
		KLSW 6229	+++	+++	+++
		KLSW 6230	+++	+++	+++
4	Chromium	KLSW 6225	+++	+++	+++
		KLSW 6226	+++	+++	+++
		KLSW 6228	+++	+++	+++
		KLSW 6229	+++	+++	+++

Table 4: Comparative growth of MRB in two different media

Sl. No.	Strain code	Log cfu of MRB		Growth of MRB	
		HMAM	Cetrimide Agar	HMAM	Cetrimide Agar
1	KLSW 6225	4.20	6.204	++	+++
2	KLSW 6226	4.20	6.204	++	+++
3	KLSW 6227	4.05	6.049	++	+++
4	KLSW 6228	4.08	6.079	++	+++
5	KLSW 6229	4.3	6.301	++	+++