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Multiple Heavy Metal and Antibiotic Tolerance Bacteria Isolated from Equatorial Indian Ocean

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Abstract: A total of twenty aerobic heterotrophic bacterial strains were isolated from collected water samples of SK-289 expedition along the cruise track of Indian Ocean Equatorial region during the month of September 2011. Amongst, six bacteria (LD5-1, LD5-2, LD5-3, LD5-4, LD5-5 and LD-10) were screened as heavy metal tolerance bacteria and tested for susceptibility to four heavy metals by using a micro dilution method for Maximum Tolerable Concentration (MTC) determinations. MTC results showed that, strain LD5-3 showed maximum resistant patterns against lead (800 mg/l), copper (700 mg/l) and zinc (400 mg/l) and less resistant to cadmium (50 mg/l) whereas strain LD-10 showed maximum resistance to cadmium (150 mg/l). The results of antibiotic susceptibility patterns revealed that metal tolerance of these isolates was also associated with resistance to antibiotics. Microbial growth in different metals displayed that cadmium metal ion mostly inhibits the growth rates of bacteria compared to other tested metals. The metal-resistance properties of these isolates are possible biotechnological tools in heavy metal bioremediation

Key words: Heavy Metal Resistant · Bacteria · Antibiotic Susceptibility · Growth Rate

INTRODUCTION

The aquatic system extends over very densely populated areas and is subject to intensive exploitation. In the past two decades, this increase in urbanization and industrialization leads to an increase of marine discharges and, therefore, the total load of pollutants being delivered to the sea [1]. Pollution of these natural environments by discharges containing heavy metals and other toxic substances is a worldwide problem as these metals are indestructible and have toxic effects on living organisms when they exceed a certain concentration limit [2]. The heavy metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium when accumulated in soils and water bodies they may be present in concentrations toxic to plants, animals, humans and aquatic life [3,4]. All organic material has to be transported to the deep sea by sedimentation from the productive upper water column, or lateral transport from the continental shelves [5].

Bacteria play a key role in all major biogeochemical cycling processes in deep-sea sediments, where metabolic activity of bacterial communities occur [6-9]. Many of the microorganisms show adaptation to the toxic materials constantly released into their environment. They have developed strategies to resist, tolerate, metabolize and to detoxify these toxic substances [10]. In recent years, extensive studies have been carried out to detect the microbes with high metal resistant and biosorption abilities from marine environments [11, 12].

An increase in the resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is due to the growth primarily of the resistant bacteria [14-17]. Plasmids are known to carry resistance to antibiotics and metals [17-19]. Hence the possibility of deep sea bacteria to harbour antibiotic/metal resistance traits via horizontal transformation can be anticipated. The aim of the present study was to isolate and identify heavy metal resistant bacteria from the water samples of Equatorial Indian Ocean as well as to determine the susceptibility to antibiotics and investigate the effect of metals on bacterial growth kinetics.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation: Water samples were collected in the sterile screw cap bottles from 10 locations (Table 1) along the cruise track (SK-289) of Equatorial Indian Ocean by using water sampler (CTD).

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Table 1: Geographical location of sampling sites

Station No.	Latitude	Longitude
1	0° 0.337'N	80° 30.359'E
2	0° 0.042'N	80° 30.032'E
3	0° 0.066'N	80° 30.059'E
4	0° 0.035'N	80° 30.126'E
5	0° 0.014'N	80° 30.077'E
6	0° 0.019'N	83° 0.077'E
7	0° 0.012'S	83° 0.034'E
8	2°59.953'S	82° 59.898'E
9	2°0.038'S	82° 59.993'E
10	5°0.152'S	82° 59.936'E

After collection, the water samples were stored immediately at 4°C until further analysis. Isolation of bacteria was conducted through primary enriching method as follows, 1ml of each water sample was added to 50 ml Nutrient broth and was incubated for 24 hours at 37°C on a shaker at 100rpm. After incubation 1 ml of medium containing cells were serially diluted up to 10^{-7} levels. 0.1 ml of the diluted suspension was spread over the surface of sea water nutrient agar medium (SWNA) and incubated at room temperature for 24-48hours [10, 11]. After incubation, the distinct bacterial colonies were picked out and purified by repeated streaking on SWNA medium. A stock culture was maintained and periodic subculture on the same culture medium was made and stored at 4°C.

Stock Solution Preparation: All regents used were of analytical grade. The 2000 mg/L of Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} stock solution was prepared by dissolving the exact quantities of the $CdCl_2 5H_2O$ (LOBA CHEMIE), $CuSO_4 \cdot 5H_2O$ (LOBA CHEMIE), $Pb(NO_3)_2$ (RANKEM) and $ZnCl_2$ (RANKEM) in deionized distilled water and were sterilized at 110°C for 15 minutes [19]. The prepared stock solutions were kept at 4°C and were used no longer than one month storage. The working concentrations of Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} solutions were prepared from the stock solutions

Screening, Characterization and Identification of Heavy Metal Resistant Bacteria: In order to screen the heavy metal resistant bacteria, the purified bacterial strains were streaked on nutrient agar plates containing 10 mg/L concentrations of heavy metals ($CdCl_2$, CuSO4•5H2O, $Pb(NO3)_2$ and $ZnSO_4$) and incubated at room temperature for 24-48 hours. The resistant bacterial strains were taken and characterized by colony morphology, staining and biochemical tests. The resistant strains were identified according to Bergey's manual of Systematic Bacteriology [20] and bacterial identification software Abis 6 online [21].

Molecular Identification of Selected Strain LD5-3: The selected LD5-3 strains were identified by determination of 16S rRNA gene sequences. Colony PCR was performed from live cells cultured on solid LB medium and the 16S rRNA were amplified by PCR using the following primers 27 f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGTACCTTGTTACGACTT-3′) [22]. The amplified DNA was sequenced in XCLERIS Biotech Pvt. Ltd., Ahmadabad, India. The nucleotide sequence obtained was compared with the known bacterial sequences available in the NCBI database using the bioinformatics tool BLAST.

Determination of Maximum Tolerance Concentrations of Resistant Bacteria: To determine the MTCs (Maximum Tolerable Concentration) for different heavy metals, bacteria were grown on 10 ml of sea water nutrient broth in the presence of different concentrations of different metals, Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} at room temperature for 24-72 hours under shaking. The MTCs refers to the highest concentration of each metal at which the bacterial growth was still observed. A control consisted of a metal-supplemented medium without the microorganisms was maintained. After incubation the tubes were observed for bacterial growth [23]. Triplicates were maintained throughout the study.

Antibiotic Susceptibility Test: Antibiotic susceptibility profile of selected metal-resistant strains was performed following disc-diffusion method [24]. The following antibiotics such as Penicillin-G (10 mcg), Ampicillin (10 mcg), Streptomycin (25 mcg), Tetracycline (30 mcg), Nalidixic acid (30 mcg) and Novobiocin (30 mcg) were used. Antibiotic impregnated discs (HI-MEDIA) were placed on Muller-Hinton agar plates inoculated with individual isolates. Plates were incubated at 37 °C for 24-48 h. The diameter of inhibition zone was recorded to nearest mm for determining the sensitivity and resistance to antibiotics. Based on inhibition zones the organisms were categorized as resistant, intermediate and sensitive as per manufacturer manual.

Determination of the Effects of Metals on Bacterial Growth: A 5 ml of mid-log phase culture of the bacterial suspensions was inoculated in 150 ml nutrient broth supplemented with 50 mg/l metal solutions (Cd, Cu and

Pb). Medium without metal but the bacterial inoculum (biotic control) and medium with metal but without bacteria (abiotic control) served as controls [25]. The initial density of the cell suspension was noted by UV-visible spectrophotometer (UV-1800). Then the flasks were incubated at room temperature on a shaker at 100 rpm. Every 6 hours, the samples were taken and measured up to 96 hours. Culture turbidity was measured as a function of time as the optical density (OD) at 600 nm on a UV-vis Spectrophotometer (UV-1800) [26].

RESULTS AND DISCUSSION

Isolation and Screening of Heavy Metal Resistant Marine Bacteria: In total, twenty different distinct bacterial colonies were isolated from deep sea water samples of 10 different sites in Indian Ocean Equator Region through primary enriching method. Amongst, six bacteria were screened as heavy metal resistant bacteria and remaining 14 strains were sensitive. This result revealed that the bacteria tolerance to heavy metal was recorded in low number in this region compared to sensitive one. The resistant bacteria were identified as *Staphylococcus* sp. (LD5-1), *Morgonella* sp. (LD5-2), *Enterobacter* sp (LD5-3), *Brennneria* sp (LD5-4), *Salmonella* sp. (LD5-5) and *Leminorella* sp (LD-10) according to Holt *et al*. [18] and bacterial identification software Abis 6 online by using morphological, staining and biochemical tests and the sensitive bacteria remained unidentified (Table 2). The heavy metals resistance profile was found in both Gram positive and Gram negative bacteria. The resistance to heavy metals in both Gram positive and negative bacteria is common phenomena in polluted environment and also reported by several researchers [11, 27].

Table 2: Screening of heavy metal resistant bacteria

Colony no	Strain name	Patterns	Identified resistant strains
1	LD1	×	UI
2	LD2-1	×	UI
3	LD2-2	×	UI
4	LD3	×	UI
5	LD4-1	×	UI
6	LD4-2	×	UI
7	LD5-1	✓	*Staphylococcus* sp
8	LD5-2	✓	*Morgonella* sp
9	LD5-3	✓	*Enterobacter* sp
10	LD5-4	✓	*Brennneria* sp
11	LD5-5	✓	*Salmonella* sp
12	LD5-6	×	UI
13	LD6	×	UI
14	LD7-1	×	UI
15	LD7-2	✓	UI
16	LD7-3	✓	UI
17	LD7-4	✓	UI
18	LD8	✓	UI
19	LD10-1	✓	UI
20	LD10-2	✓	*Leminorella* sp

✓-Resistant; ×-Sensitive; UI-Unidentified;

Among the resistant bacteria, Gram negative bacteria were dominant compared to Gram positive bacteria. The sequence analysis (using the BLAST database of the National Center for Biotechnology Information; [http://www.ncbi.nlm.nih.gov]) LD5-3 belonged to the *Enterobacter* genus, showing a high 16S rRNA gene sequence similarity (99%) to *Enterobacter asburea.* The sequences of strain LD5-3 matched 99 % with 16S rRNA of the *Enterobacter asburiae* strain M-T-MRS_78 and *Enterobacter* sp. WS05. These sequences were submitted to National Centre for Biotechnical Information and retrieved with accession number (GenBank ID: KC148529) (Figure 1).

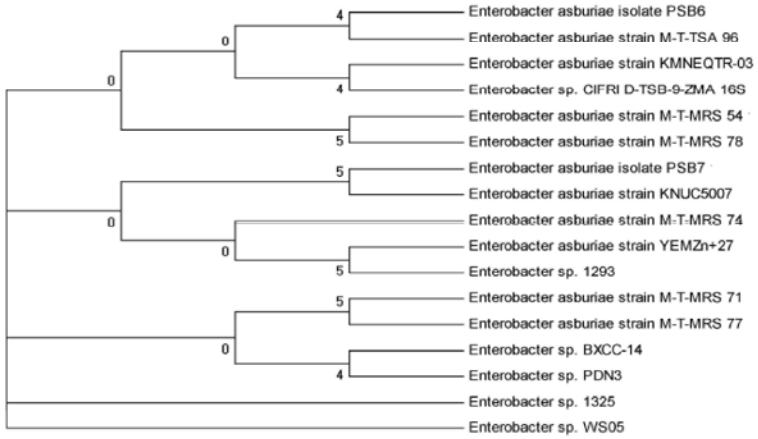

Fig. 1: A Phylogenetic tree constructed based on 16s rRNA of *Enterobacter asburiae* strain KMNEQTR-03 and reference strains by the neighbour joining method using MEGA. 5.0 software

Table 3: Maximum tolerance concentrations (mg/L) of the resistant strains

	Resistant strains					
Metal ion	LD5-1	LD5-2	LD5-3	LD5-4	LD5-5	LD-10
Cu^{2+}	500	600	700	600	600	600
Cd^{2+}	50	50	50	100	50	150
Pb^{2+}	600	700	800	500	700	700
Zn^{2+}	200	200	400	100	200	300

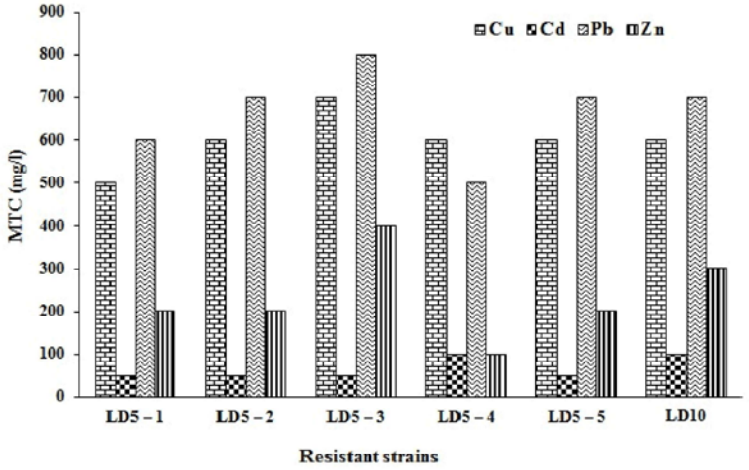

Fig. 2: Maximum tolerable concentration (mg/L) of resistant isolates against various metals.

Determination of Maximum Tolerance Concentrations of Resistant Bacteria: The result showed that strain LD5-3 has highest MTC value for Pb^{2+} (800 mg/L), Cu^{2+} (700 mg/L) and Zn (400 mg/L) whereas LD-10 has highest MTC value for Cd^{2+} (150 mg/l). Following, the strain LD5-4 has MTC value for Cu^{2+} (600 mg/l), Pb^{2+} (500 mg/l) and Zn^{2+} (100 mg/l) and Cd^{2+} (100 mg/l). The low MTC of cadmium value was noted in the strains of LD5-1, LD5-2, LD5-3 and LD5-5 is 50 mg/l whereas, maximum MTC of cadmium were recorded in strains of LD5-4 (100 mg/l) and LD-10 (150 mg/l). The maximum MTC value of Zn was recorded in the strains of LD5-3 whereas; the maximum MTC value of Pb^{2+} was recorded in LD5-3 (800 mg/l) (Table 3). The strain LD5-3 had also maximum MTC value of Cu^{2+} (700 mg/l). In this study all the strains showed resistance in the order of metal concentrations of $Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$ except LD5-4 (Fig. 2). This varying response of selected bacteria might be due to the difference in their cell wall composition or due to variations in resistance mechanisms [28].

Antibiotic Susceptibility Test: Antibiotic susceptibility patterns of resistant isolates were shown in table 4. The isolates exhibited a varied degree of sensitivity and resistance to different antibiotics. The LD5-1and LD5-4 isolates were found to be susceptible to 4 antibiotics and displayed intermediate susceptibility to 2 antibiotics. The isolates LD5-2 showed resistance to Penicillin (10 units), Streptomycin (25 mcg) and Novobiocin (30 mcg), whereas it showed intermediate susceptibility to Ampicillin (10 mcg), Tetracycline (30 mcg) and Nalidixic Acid (30 mcg). The isolates LD5-5 were resistant to Ampicillin (10 mcg) and sensitive to other antibiotics. The results of this study revealed that all the strains showed sensitivity against at least one or two antibiotics. In the present study, high degree of heavy metal resistance associated with multiple antibiotic resistance were detected in isolates LD5-2 and LD5-5 isolated from equatorial regions of Indian Ocean. Even though the isolate LD5-3 was highly resistant to heavy metal, it was sensitive and intermediately sensitive to most of the tested antibiotics except Penicillin. Similar patterns were observed in LD-10. The antibiotic resistance in metal tolerant marine bacterial population was also observed in previous studies [29]. An increase in the resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is due to the growth primarily of the resistant bacteria [13-17]. Many earlier studies observed that heavy metal resistant bacteria are also resistant to many antibiotics and other toxic chemicals [30-32] by virtue of carrying plasmids and

Table 4: Antibiotic susceptibility patterns of metal resistant strains isolated from equatorial regions of Indian Ocean

		Resistant strains					
Antibiotics	Disc content	LD5-1	LD5-2	LD5-3	LD5-4	LD5-5	LD-10
Ampicillin (A)	10 mcg	30 (S)	18 (IM)	19 (S)	16 (IM)	NZ (R)	16 (IM)
Penicillin (P)	10 units	30 (S)	8 (R)	NZ (R)	18 (S)	15 (S)	8 (R)
Streptomycin (S)	25 mcg	17 (IM)	NZ (R)	13 (IM)	18 (S)	22 (S)	22 (S)
Tetracycline (T)	30 mcg	24 (S)	14(IM)	13 (IM)	34 (S)	26 (S)	22 (S)
Nalidixic Acid (NA)	30 mcg	17 (IM)	18 (IM)	19 (S)	16 (IM)	NZ (R)	16 (IM)
Novobiocin (NV)	30 mcg	26 (S)	9(R)	NZ (R)	38 (S)	18 (IM)	18 (IM)

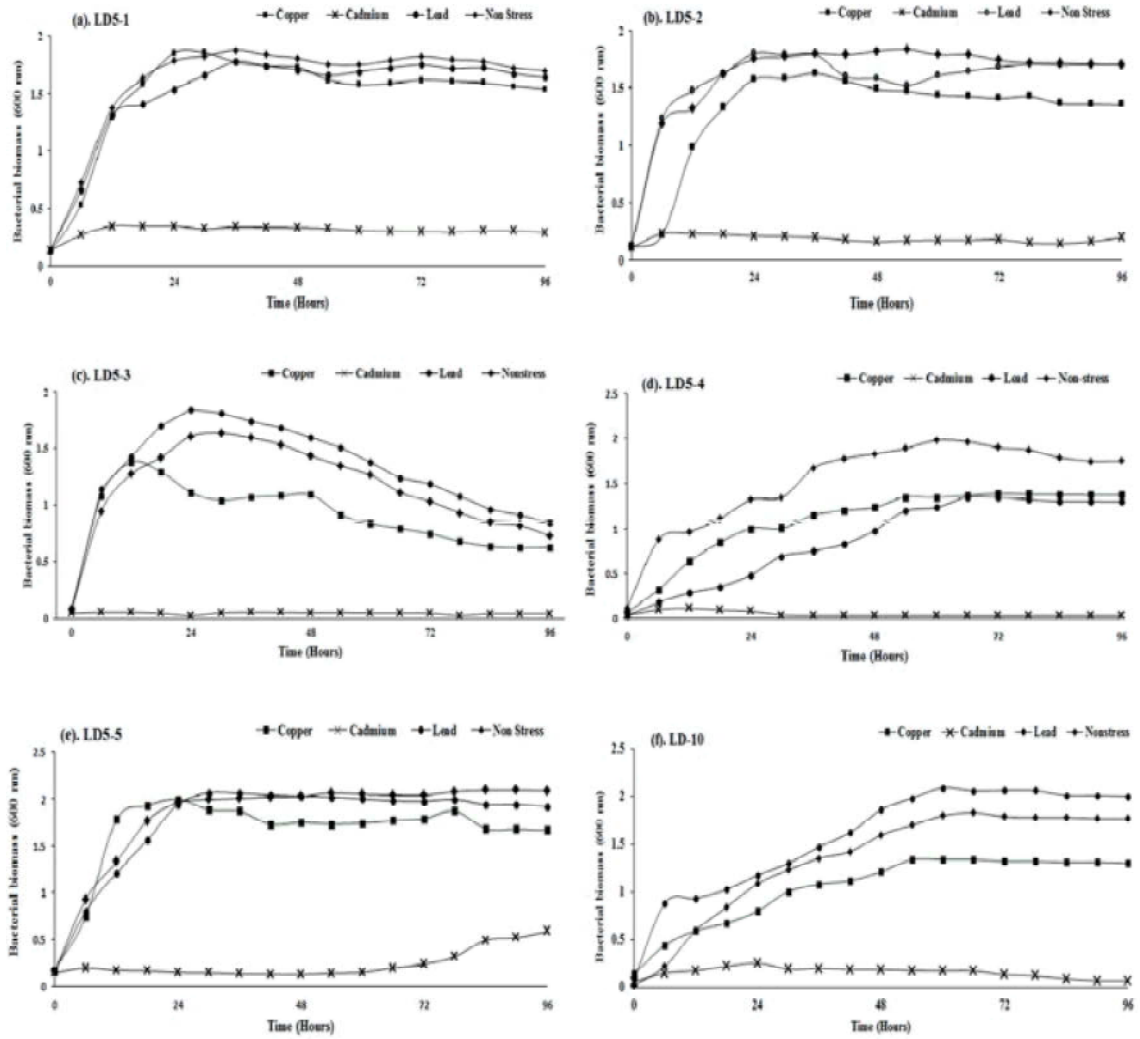

Fig. 3: Growth rates of resistant isolates in various heavy metals. (a) LD5-1, (b) LD5-2, (c) LD5-3, (d) LD5-4, (e) LD5-5 and (f) LD-10.

or transposons encoding genetically linked metal and antibiotic resistance. Ecological studies have reported that metal and antibiotic resistance is becoming a global phenomenon, with the equatorial region waters not being exempted, as the frequency of occurrence of plasmid-borne bacteria was high in deep sea region [34]. Plasmids are known to carry resistance to antibiotics and metals [15, 17, 18]. Hence the possibility of deep sea bacteria to harbor antibiotic/metal resistance traits via horizontal transformation can be anticipated.

Determination of the Effects of Metals on Bacterial Growth: The growth response of metal resistant bacteria in different metal treated concentrations was illustrated in figure 3 (a) to 3 (f). The strains of LD5-3 and LD5-4 growth rates was inhibited hardly when inoculated into the 50 mg/l cadmium treated SWN broth whereas strain of LD5-1, LD5-2, LD5-5 and LD-10 growth rates was very slow. During the growth study, the colour of lead treated SWN broth was changed to black colour due to formation of lead sulphide in the medium. However, all the isolates produced sulphide in lead treated SWN broth. All the strains reached log phase in 6 hours and reached stationary phase after 18 hours in metal treated condition excluding cadmium. Growth response in metal treated condition is mainly depends on maximum metal tolerance concentration of bacteria [35]. The long lag phase observed in the metal treated condition is probably due to the adaptation of the bacteria to the metal treated environment [36]. Among the tested metals, cadmium hardly affected the bacterial growth rates when compared to other metals. The growth curve patterns of resistant isolates were entirely different based on the tested metals. Particularly bacteria growing in non-metal condition showed outstanding growth rates compared to metal treated condition [37].

The current work demonstrated that the tolerance of heavy metals varied between bacteria even though they were isolated from the marine water sample. Among all the isolates LD5-3 showed resourceful tolerance against most of the heavy metals used. The resistance of these marine bacteria to several heavy metals enthuses to affirmatively recommend their potential to be exploited in bioremediation of heavy metals. Further investigation on marine heavy metal resistant bacteria may lead to new and better understanding of the existing concept.

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