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Detection of heavy metal resistance bioluminescence bacteria using microplate bioassay method

P. Ranjitha[1] and E.S. Karthy[2]

[1]Department of Biotechnology, University of Madras, Chennai-25, Tamilnadu, India.

[2]AWECARE, Analytical & Research Laboratories, Postal Nagar, Erode- 638 011, Tamilnadu, India.

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ABSTRACT

Effect of different heavy metals on *Vibrio harveyi*, *V. fischeri*, *Photobacterium phosphoreum* and *P. leiognathi* were examined. Checkerboard assay used for the detection of the natural metal tolerance levels of a large number of marine luminous eubacteria. In this purpose, we surveyed 57 strains of luminous bacteria for their natural patterns of heavy metal tolerance. The behaviors of these strains were not homogeneous with respect to all metals tested, even within the strains belonging to the same genus. At least 1 to 4 different MICs were detected for every metal except barium and cobalt. Isolated bacteria were tested for the presence of plasmids using the modified alkaline lysis method was effective for identification of plasmids of different sizes. This study revealed that the frequency of the occurrence of plasmids in heavy metal resistance bacteria and suggested that plasmids are highly ubiquitous and predominant in most heavy metal resistant bacteria.

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Introduction

Heterotrophic aerobic marine bacteria are found in all oceans, semi closed seas and brackish coastal lagoons. These organisms respond rapidly to environmental modifications and their abundance and composition may provide an indication of the breathing of oceans[1]. Bacteria are known to concentrate and excrete varieties of metal ions[2]. There are several studies on the effects of heavy metals on the growth of different species of bacteria[3], but there are no such studies on bioluminescent bacteria[4]. But in the year 1993, Ramaiah and Chandramohan found that the ion can drastically reduce luminous bacterial growth[5]. Bacterial resistance to heavy metals is widespread. In one investigation, twenty five heavy metal resistance strains were isolated from the Alzahra Hospital of Isfahan in Iran. Most of the isolated bacteria demonstrated multiple resistances to four heavy metals (Hg, Cu, Ph, and Cd). In most of the 25 isolated strains showed plasmid of different molecular size[6].

The test was performed using the 96 well Microplate based broth dilution method, also known as checkerboard or Microplate assay[7]. Microplate method proved to be an easy to use and effective tool in the identification of antibiotic resistance bacteria. In the presence study used the same method for detection of Heavy metal resistant bacteria even to a lower concentration level. The method takes benefits of a known working assay and combines it with the ease of interpretation provided by the color indicator, which can be used successfully with several strains. Advantages over the agar dilution method include increased sensitivity for small quantities of heavy metals, ability to quantitative determination of MIC. We believed that this method will be use to many researchers in the field of identification and evolution of heavy metal resistant bacteria in toxicology study in the marine/other environment. The presence study also deals with isolation of heavy metal resistance bioluminescence bacterial plasmids from the South and East coast of Tamilnadu. The data obtained provide clues regarding the prevalence of plasmids in heavy metal resistant bioluminescence bacterial populations residing in the marine.

Materials and Methods

Collection of Marine Samples

Three sampling stations representing different marine environment were chosen in East and South coast of Tamilnadu, India. Marine samples were collected from various places: i) Raimanthurai, Thankai Pattinam, Colachael, Manavalakurichi, Chinamuttam (yard), Manakudi, Nagarkoil of Kaniyakumari district. ii) Cuddalore (OT), Pichavaram, Poombukar, Parangipettai of Cuddalore district. iii) Veerampatinam, Chinna Veerampatinam of Puducherry union.

Isolation and Identification

Luminous bacterial strains were isolated by transport the samples within 6 hours of harvest and partially submerged with artificial sea water (0.4M NaCl, 0.1M $MgSO_4$, 0.02M KCl, 0.02M $CaCl_2$) except sea water and sediments samples were serially diluted for plating. The partially submerged fish, shrimp, squid, crab, lobster were stored at 28º C for 2-3 days and inspected visually for the presence of luminous areas daily. Luminous area were swabbed and purified into pure culture in Sea Water Complete (0.38M NaCl, 0.02M $MgCl_2$, 0.25M $MgSO_4$, 8mM KCl , 0.5% peptone, 0.3% yeast extract and glycerol, 2% agar) agar plates. The luminous bacteria Identified based on the work of Reichelt and Baumann[8].

Heavy metal toxicity test–checkerboard assay

Preparation of Bacterial Suspension

A single colony of bacteria in SWC broth was incubated overnight at 30ºC. The bacterial suspension was centrifuged at 4000 rpm for 5 min.

The cells were suspended in normal saline for 3 times. After the last run, the bacteria were resuspended in 20 ml of normal saline (0.9% NaCl w/v). The suspension wasdiluted to a final concentration of $5x10^6$ CFU/ml was calculated.

Tele:

E-mail addresses: ranjithaponn@gmail.com

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Preparation of Microtiter Plate

Column one to seven and the rows A to L of a microtiter plate were marked off. The wells 1-7 of row A, the 100 µl metal ions (mercury (Hg), Silver (Ag), Zinc (Zn), Barium (Ba), Lead (Pb), Copper (Cu), Cobalt (Co)) were added at a concentration of 40mM. 50 µl of sterile normal saline was added to rows B-L. The undiluted heavy metals from the row A were mixed using a multi channel micropipette with tips attached to consecutive channels. From the row A, 50 µl each was transferred to the wells of row B. Tips were discarded to minimize carry over and new tips were attached to multi-channel pipette contents of row B were thoroughly mixed. The above process was repeated until 12 serial dilutions. 40 µl of double strength SWC broth and 10 µl of bacterial suspension at a 5x 10^6 concentration were added to each well. To prevent dehydration the plates were covered with sterile plastic cover and then incubated at 30 ºC for 24 hrs. The plates were examined for growth or inhibition after 12 to 24 hrs by adding 40 µl of Diphenyl (i-naphthyl) tetrazolium chloride (DTC) and plates were incubated additional 30 min to 2 hrs. The MIC was determined the lowest sample concentration at which no violet color (significant live growth) appeared. The assay was performed several times in triplicate using all strains. The lowest concentration of metal that completely prevented growth was termed the MIC.

Plasmid isolation

The presence of plasmids in the marine isolates was determined using a modification of alkaline lysate method[9,10]. Plasmid DNA was separated by electrophoresis on a 0.8% agarose gel (w/v) at 50 volts. Lamda *Eco* R1 was used in each gel as molecular marker. The gel was stained with ethidium-bromide, visualized under UV transillumination and photographed.

Results and discussion

Eighty five specimens were collected and quadruplicate replicates of duplicate of each seawater, sediment, fish, shrimp, squid, crab, lobster samples to minimize sample variance arising from sample processing. Luminous strains of marine bacteria isolated off coast of Tamilnadu were subjected to phenotypic characterization. Luminous marine isolates were readily identified by application of a relatively few simple 33 diagnostic traits. Of the many thousands of luminous colonies that appeared on isolation plates, 57 were selected randomly. No duplicate isolates were selected from single specimen. Of the 57 luminous bacterial isolates obtained in the pure culture, 33 were *V. fischeri, 13* were *P. phosphoreum,* 8 were *V. harveyi* and 3 were *P. leiognathi* (Table 1). These species constituted 58, 23, 14 and 5% respectively.

In checkerboard assay the concentration of 40mM was used as an initial dose for metal ions. Then it was double diluted up to 0.01mM. The 57 strains of luminous species showed most homogeneous behavior in tolerance level for barium and cobalt. The four species of luminous bacteria exerted a very heterogenous behaviour for individual susceptibility levels of mercury, silver, zinc, lead and copper. *V. fischeri, P. phosporeum, V. harveyi* and *P. leiognathi* showed equal susceptibility and lowest MICs to mercury and zinc when compared to remaining metal ions. Most of the strains showed MIC of 0.025 for zinc, 1.0 for lead and silver and 2.5 for copper. The table 2 and 3 shows the percentage of tolerance of the luminous bacteria for 7 metal ions. Minimal inhibitory concentration of luminescence also same of the MICs of luminous bacterial growth. The luminous species showed pronounced tolerance to barium and cobalt. Inhibition of growth of different luminous strains by various heavy metal ions was in the following order: Mercury>Zinc>Copper>Silver>Cobalt >Barium.

All the fresh isolates showed uniform tolerance of chromium, nickel and lead and an equal sensitivity to silver and mercury[11]. The *Vibrio costicola* strains showed a general pattern of tolerance or sensitivity of moderately halophillic eubacteria to these heavy metals. However, *V. costicola* exhibited a higher sensitivity to copper and cobalt and with respect to cadmium, the response was similar to that found for moderately halophilic cocci[12].

Generally all the heavy metals decreased the light of luminous bacteria. 2.5 to 5 mg 1^{-1} of ion can drastically reduce luminous bacterial growth, concentrations as low as 0.1 mg 1^{-1} of Hg, Cu, Cd or 2.5 mg 1^{-1} of Ni and Pb were found to inhibit luminescence completely. It is highlighted that this system is lost irreversibly in these bacteria grown in the presence of these ions and those occurring in polluted waters[5]. Determination of the ratio of bioluminescence to total platable bacteria (Bioluminescnt Ratio-BLR) is a potentially attractive indicator system for coastal systems since it targets native microorganisms and is a simple low cost assay that does not require the use of sophisticated equipment[13].Despite their ecological significance, it was only recently suggested that aerobic heterotrophic bacteria, with their luminescent component, might be used as indicators of coastal water quality[1]. The copper is modified in the presence of agar and also reported that although currently no alternative procedure has been adopted by the scientific community[14,11]. The above problem was overcome by this checkerboard assay.

The relative migrations of unknown plasmids were estimated by the use of molecular weight marker λ/EcoRI, 36 bacterial strains have plasmids with a large size molecular weight 21,226 bp. Three strains showed plasmids with a molecular weight between 5,643 bp and 4,878 bp, 54 strains have plasmids with molecular weight smaller than 3,530 bp. The 36 isolates were represented large plasmid size and small plasmid size contemporaneously. Most of the heavy metals resistance bacteria were found to be carrying plasmids.

The frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria. Most plasmid carrying bacteria in the marine strains were obtained from Delftia strains that carry plasmids of big and small sizes[15]. Fifty environmental isolates of *Vibrio* species were isolated from water samples of Mai Po Nature Reserve and the Cape d'Aguilar Marine Reserve in Hong Kong and screened for the presence of plasmid. Their result shows that the high frequency of plasmid in *Vibrio* species of both polluted and pristine environments are ecologically important to the survival of these bacteria in the environment. The ecological function and evolutionary role of plasmids remain to be elucidated[16].

Bacteria isolated from industrial waste water had highest plasmid incidence 48% followed by sediment 30% and those from sea water had lowest plasmid incidence 22%[17,18,19]. Plasmid in natural populations of marine bacteria was 23% in an unpolluted site of the Gulf of Mexico, 46% in Chesapeake Bay, 43% (marine luminous bacteria) in Mediterranean and Red sea and 28% in the Antartic[20].

Conclusion

This study concluded that the *Vibrio* species are widely occurring in the marine environments and a significant portion

of the bacterial isolates showed heavy metal resistance in checkerboard assay. Most of them may have multi plasmids. These plasmids are responsible for their survival through resistant to high concentrations of pollutants.

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Table 1: Percentage of Bioluminescence Bacteria in Different Marine Environmental Sources

S.No	Types of Samples N=85	LB+ (%)	LB – (%)	Bioluminescence Bacteria (%)				
				VF	VH	PP	PL	Total No. of Strains
1	Sea Water (SW) N=16	11 (69)	5 (31)	9 (56)	1 (6)	-	1 (6)	11 (68)
2	Sediment (S) N=18	17 (94)	1 (6)	7 (39)	2 (11)	8 (44)	-	17 (94)
3	Fish (F) N=35	20 (57)	15 (43)	11 (31)	2 (5.7)	5 (14)	2 (5.7)	20 (56.4)
4	Squid (Sq) N=6	6 (100)	-	6 (100)	-	-	-	6 (100)
5	Crab (C) N=2	-	2 (100)	-	-	-	-	-
6	Shrimp (Sh) N=5	3 (60)	2 (40)	-	3 (60)	-	-	3 (60)
7	Lobster (Lo) N=2	-	2 (100)	-	-	-	-	-
Total No. of Strains		57 (67)	28 (33)	33 (58)	8 (14)	13 (23)	3 (5)	

VF:*V.fischeri*, PP:*P.phosphoreum* , VH:*V.harveyi*, PL:*P.leiognathi*, LB+:positive for bioluminescence bacteria, LB¬¬– :negative for bioluminescence bacteria.

Table 2: Minimal Inhibition Concentration (MIC) of Heavy Metals for Luminous Bacteria

S.No	Strains	HEAVY METAL IONS (mM)						
		$HgCl_2$	$AgNo_3$	$ZnCl_2$	$BaCl_2$	$PbNo_3$	$CuSo_4$	$CoNo_3$
1	VF 1	0.25	1.0	0.1	40	2.5	2.5	1.0
2	VF 2	0.25	1.0	2.5	40	2.5	1.0	1.0
3	VF 3	0.05	1.0	0.025	40	2.5	1.0	1.0
4	VF 4	0.25	1.0	0.025	40	1.0	1.0	1.0
5	VF 5	0.25	1.0	0.025	40	2.5	2.5	1.0
6	VF 6	0.025	1.0	0.025	40	2.5	1.0	1.0
7	VF 7	0.025	1.0	0.025	40	2.5	1.0	1.0
8	VF 8	0.05	1.0	0.025	40	1.0	1.0	1.0
9	VF 9	0.025	1.0	0.025	40	1.0	2.5	1.0
10	VF 10	0.05	1.0	0.05	40	1.0	2.5	1.0
11	VF 11	0.025	1.0	0.025	40	1.0	2.5	1.0
12	VF 12	0.05	1.0	0.05	40	1.0	2.5	1.0
13	VF 13	0.05	1.0	0.05	40	2.5	2.5	1.0
14	VF 14	0.025	1.0	0.025	40	2.5	1.0	1.0
15	VF 15	0.025	0.05	2.5	40	1.0	1.0	1.0
16	VF 16	0.05	1.0	0.05	40	2.5	2.5	1.0
17	VF 17	0.05	1.0	0.05	40	2.5	1.0	1.0
18	VF 18	0.05	1.0	0.025	40	2.5	2.5	1.0
19	VF 19	0.05	0.05	0.05	40	1.0	2.5	1.0
20	VF 20	0.025	1.0	0.025	40	2.5	1.0	1.0
21	VF 21	0.025	1.0	0.025	40	2.5	1.0	1.0
22	VF 22	0.025	1.0	0.025	40	1.0	1.0	1.0
23	VF 23	0.025	1.0	0.025	40	1.0	1.0	1.0
24	VF 24	0.05	1.0	0.1	40	1.0	1.0	1.0
25	VF 25	0.025	1.0	0.025	40	1.0	1.0	1.0
26	VF 26	0.05	1.0	0.05	40	1.0	1.0	1.0
27	VF 27	0.025	1.0	0.025	40	1.0	2.5	1.0
28	VF 28	0.025	1.0	0.025	40	2.5	1.0	1.0
29	VF 29	0.05	1.0	0.1	40	2.5	1.0	1.0
30	VF 30	0.05	1.0	0.05	40	2.5	2.5	1.0
31	VF 31	0.05	1.0	0.025	40	2.5	2.5	1.0
32	VF 32	0.025	1.0	0.025	40	2.5	1.0	1.0
33	VF 33	0.025	1.0	0.025	40	2.5	1.0	1.0
34	PP 1	0.05	1.0	0.05	40	1.0	1.0	1.0
35	PP 2	0.025	1.0	0.025	40	2.5	2.5	1.0
36	PP 3	0.025	1.0	0.025	40	1.0	2.5	1.0
37	PP 4	0.025	1.0	0.025	40	2.5	1.0	1.0
38	PP 5	0.025	1.0	0.025	40	2.5	1.0	1.0
39	PP 6	0.05	1.0	0.025	40	1.0	1.0	1.0
40	PP 7	0.025	1.0	0.025	40	1.0	1.0	1.0
41	PP 8	0.025	1.0	0.025	40	1.0	1.0	1.0
42	PP 9	0.025	1.0	0.025	40	2.5	1.0	1.0
43	PP 10	0.025	1.0	0.025	40	2.5	2.5	1.0
44	PP 11	0.025	1.0	0.025	40	2.5	2.5	1.0
45	PP 12	0.025	1.0	0.025	40	2.5	1.0	1.0
46	PP 13	0.025	1.0	0.025	40	2.5	2.5	1.0
47	VH 1	0.05	1.0	0.025	40	2.5	2.5	1.0
48	VH 2	0.025	1.0	0.025	40	1.0	1.0	1.0
49	VH 3	0.025	1.0	0.025	40	1.0	2.5	1.0
50	VH 4	0.025	1.0	0.025	40	1.0	2.5	1.0
51	VH 5	0.025	1.0	0.025	40	1.0	2.5	1.0
52	VH 6	0.025	1.0	0.025	40	2.5	1.0	1.0
53	VH 7	0.025	1.0	0.025	40	2.5	2.5	1.0
54	VH 8	0.025	1.0	0.025	40	2.5	1.0	1.0
55	VL 1	0.025	1.0	0.025	40	1.0	1.0	1.0
56	VL 2	0.025	1.0	0.025	40	2.5	1.0	1.0
57	VL 3	0.025	1.0	0.025	40	1.0	2.5	1.0

VF:*V.fischeri*, PP:*P.phosphoreum* , VH:*V.harveyi*, PL: *P.leiognathi*.

Table 3: Percentage Comparison of Minimal Inhibition Concentration (MIC) for Metal Ions

S. No	Isolates	MIC/No. of strains[a] (%)						
		$HgCl_2$	$AgNo_3$	$ZnCl_2$	$BaCl_2$	$PbNo_3$	$CuSo_4$	$CoNo_3$
1	VF	0.25/4 (12) 0.05/14 (42) 0.025/15 (45)	1/31 (94) 0.05/2 (6)	0.1/3 (9) 2.5/2 (6) 0.025/20 (60) 0.05/8 (24)	40/33 (100)	2.5/19 (58) 1.0/14 (42)	1.0/20 (61) 2.5/13 (39)	1.0/33 (100)
2	PP	0.025/11(85) 0.05/2 (15)	1.0/13 (100)	0.025/12 (92) 0.05/1 (8)	40/13 (100)	1.0/5 (38) 2.5/8 (62)	2.5/5 (38) 1.0/8 (62)	1.0/13 (100)
3	VH	0.25/7 (88) 0.05/1 (12)	1.0/8 (100)	0.025/8 (100)	40/8 (100)	2.4/4 (50) 1.0/4 (50)	2.5/5 (63) 1.0/3 (37)	1.0/8 (100)
4	PL	0.025/3 (100)	1.0/3 (100)	0.025/3 (100)	40/3 (100)	1.0/2 (67) 2.5/1 (33)	1.0/2 (67) 2.5/1 (33)	1.0/3 (100)

MIC: Minimal Inhibition Concentration, a: Number of strains showed same MIC value.
VF:*V.fischeri*, PP:*P.phosphoreum* , VH:*V.harveyi*, PL:*P.leiognathi*,