*Available online at* www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2430

**ISSN: 2320 – 7051**

*Int. J. Pure App. Biosci.* **5 (2):** 1199-1206 (2017)

*Research Article*

International Journal of Pure & Applied Bioscience

OPEN ACCESS

# Horizontal Transfer of Heavy Metal Resistance Plasmid from A Brahcish Waster Bacterium *Pseudomonas* Sp. AMET1221 to *Escherichia coli* DH5ὰ

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Received: 29.12.2016 | Revised: 10.01.2017 | Accepted: 12.01.2017

**ABSTRACT**

*A total of forty eight heavy metal resistant bacterial strains were isolated from collected water samples of Kanathur Beach and Buckingham Canal at Kanathur, TamilNadu, India. Based on the minimum inhibitory concentration (MIC) of the strain toward chromium and mercury, resistance was found to be 50µM and 10mM respectively. MIC determinations amongst four strains (AMET1217, AMET1221, AMET1229 and AMET1242) were screened as highest heavy metal resistance bacteria. The present study proved the genetic contribution of heavy metal resistance in this strain to be plasmid mediated. Plasmid curing experiments affirmed plasmid mediated heavy metal resistance. Additionally, genetic transformation of a non metal resistant lab strain Escherichia coli and the cured strain of Pseudomonas sp. AMET1221with the isolated plasmid increased their metal tolerance level and confirming the genetic determinant to be present in the plasmid. The metal-resistance properties of these isolates are possible biotechnological tools in heavy metal bioremediation.*

***Key words:*** *Biotransformation, Environmental Pollution, Metal Resistance, Plasmid curing*

## 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[4]. 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[11].

**Cite this article:** Jayaprakashvel, M., Bharathi, C.D., Muthezhilan, R. and Hussain, A.J., Horizontal Transfer of Heavy Metal Resistance Plasmid from A Brahcish Waster Bacterium *Pseudomonas* Sp. AMET1221 to *Escherichia coli* DH5ὰ, *Int. J. Pure App. Biosci.* **5(2):** 1199-1206 (2017). doi: http://dx.doi.org/10.18782/2320-7051.2430

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[1,13]. 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[4].

Elevated levels of heavy metal not only decrease soil microbial activity, crop production but also threaten human health through food chain. Microorganisms are first biota that undergoes direct and indirect impact of heavy metals being in available forms in soil solutions or absorbed on soil colloids. Microbial survival in polluted soil depends on intrinsic biochemical and genetic adaptation including morphological changes of cell as well as environmental modification of metal speciation. Many microorganisms naturally possess the ability to inhabit metal-polluted areas. An advanced understanding of the mechanisms by which microbes cope with metal stress will facilitate the rational design of strategies for detection and bioremediation of heavy metal-polluted water and soil systems. When the bacterial cells are exposed to the high concentrations of heavy metals, the metals react within cells with various metabolites and form toxic compounds. Mechanisms for uptake of these metal species are present in the bacterial cell through which heavy metals enter the cell[10]. 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[17]. An increase in the resistant fraction of culturable heterotrophic bacteria in the aquatic ecosystems is due to the growth primarily of the resistant bacteria[16]. Plasmids are known to carry resistance to antibiotics and metals[20].

Microbes apply various types of resistance mechanism in response to heavy metal. The mechanism may be encoded by chromosomal genes. However most resistant system appears to be associated with plasmid. Plasmids are extra chromosomal piece of DNA which has capability to replicate independently of the host chromosome. Various phenotypic character encoded by plasmid; these include antibiotic and metal resistance, degradation of complex organic compounds, production of enterotoxins and colicins, production of restriction enzyme. Genetic interactions among bacteria are mediated by one of the three distinct gene-exchange mechanisms: conjugation, transformation or transduction[5]. The aim of the present study was to isolate and identify heavy metal resistant bacteria from the water samples of Indian Coastal area of Tamil Nadu, India.

## MATERIALS AND METHODS

### Collection and Isolation of Bacteria from the Water Samples

The water samples both from Kanathur Beach and Buchingham Canal at Kanathur, TamilNadu, India were collected in sterile containers and brought to the laboratory and used for the isolation of microorganisms immediately. Serial dilution plating technique was followed to isolate bacteria and nutrient agar (NA) was used as the medium for the isolation of bacteria. Samples were serially diluted up to 10-5 and 0.1 mL sample from respective dilutions were taken and used for spread plate method. The plates were then incubated for 3 days and the bacterial growth was observed at 24 h interval. The number of colonies and colony characteristics were also recorded. After three days distinct pure colonies of different morphology were isolated and subcultured in nutrient agar. The bacteria were preserved in sterile distilled water at 4ºC.

### Heavy Metal Tolerance and Maximum Inhibition Concentrations Spectrum

In order to screen the heavy metal resistance bacteria, the purified bacterial strains were streaked on NA plates amended with three different concentrations (12.5, 25 and 50 µM) of two heavy metals viz., chromium ($K_2Cr_2O_7$) and mercury ($HgCl_2$). The NA plates without heavy metals served as controls. The plates were incubated at room temperature for 24 h and observed for bacterial growth. The

resistance bacterial strains were taken and characterized by colony morphology, staining and biochemical tests. The resistance strains were identified according to Bergey's manual Systematic Bacteriology[7]. The MICs 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 were maintained. After incubation the tubes were observed for bacterial growth[18]. Triplicates were maintained throughout the study. Based on their degree of tolerance and only 4 strains designated as AMET1217, AMET1221, AMET1229 and AMET1242 were selected to further study on the plasmid mediated mercury resistance. The isolated strains were deposited and preserved to the Microbial Culture Collection Unit, Department of Biotechnology, AMET University, Tamil Nadu, India.

**Curing of Plasmids**

The four isolates that exhibited highest level of mercury resistance upto 10 mM concentration were subjected to plasmid curing experiment to confirm the plasmid mediated heavy metal resistance. Acridine orange was used as plasmid curing agent. Acridine orange dye was prepared at the concentration of 10mg/10mL. Nutrient broth (NB) was prepared and amended with acridine orange to a final concentration of 400 µg/mL and sterilized. A similar set of NB without acridine orange was also prepared and sterilized. They were labeled as acridine amended (AO+) and acridine non amended (AO-). After autoclaving and cooling, 150µL from each of the four selected strains were transferred to these acridine orange amended and non amended tubes. The tubes were incubated in the environmental shaker at 35ºC under 175 rpm for 24 h [6].

**Demonstration of Plasmid Mediated Mercury Resistance**

After 24 h the bacterial suspension from both AO+ and AO- tubes were taken in inoculation loop and replica plated on both 10 mM $HgCl_2$ amended and non amended nutrient agar medium. The colonies which failed to grow in $HgCl_2$ amended plates were assumed that they lost their plasmid in the curing process and hence the mercury resistance harbored by them is plasmid mediated.

**Plasmid Transfer Experimentsȧȧȧ**

The recipient strain *Escherichia coli* DH5α, a plasmid less strain which is highly susceptible to mercury even at 15 µg/mL concentration was obtained from Biocontrol and Microbial Metabolites Laboratory, CAS in Botany, University of Madras, India. The strain was already used in several molecular biology experiments as a model strain. Both the donor *Pseudomoas* sp. AMET1221 and recipient Escherichia coli DH5α, were grown in NB at room temperature for 12 h. Both the bacterial growth were adjusted with sterile distilled water to give 0.3 OD at 600 nm which approximately contained 106 CFU/mL. Equal volume of both the donor and recipient bacterial suspensions were taken in a sterile eppendorf tube and mixed well. Then this donor recipient mixture was inoculated in nutrient broth. The mixtures were kept in room temperature for 12 h. Then, the donor recipient mixture from each treatment was serially diluted up to 10-4 and plated on both nutrient agar and EMB agar (HiMedia, Mumbai) supplemented with $HgCl_2$ (10 mM) [14]. The number of metallic sheen E. coli colonies that were observed in mercury amended EMB agar plates is expressed as the number of transformed E. coli cells. Because, the frequency of transformation was calculated using the following formula.

Frequency of transformation = (Number of colonies in EMB agar with $HgCl_2$)/(Number of colonies in EMB agar without $HgCl_2$)

## RESULTS AND DISCUSSIONS

**Isolation of bacteria from the water samples**

A total of 48 bacterial strains were isolated from six water samples collected from Kanathur, Chennai, India. Out of 48 isolates 19 strains were isolated from 3 sea water samples collected from Kanathur Beach. Remaining 29 strains were isolated from 3 brackish water samples collected from Buchingham Canal in Kanathur. Kamalakannan et al.,[8] studied on Pulicate lake

sediments were often severely polluted with mercury compounds and other toxic metal and several mercury resistant bacteria were isolated and identified from these sediments. Enrichment and isolation of methyl chloride utilizing bacteria from a variety of terrestrial, freshwater, estuarine, marine environment resulted in detection of 6 new methyl chloride utilizing hyphomicrobium strain[15]. Based on the morphological and biochemical study the isolated heavy metal resistance bacteria 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[3].

**Heavy metal tolerance and Minimum Inhibitory Concentration**

Bacterial heavy metal tolerance spectrum was carried to two heavy metals such as Mercury and Chromium; it has been found that all the bacteria were tolerant to both the heavy metals at lower concentrations. For metal resistance profile, MIC was determined in the varying concentration of metals, the growth of the bacterial strains was observed as compared to control bacterial strain of the same. Among all the chromium tolerant bacterial isolates are exhibited upto 50 µM of $K_2Cr_2O_7$.

Mercury tolerance were tested and found that very few bacteria have tolerated mercury upto 1 mM concentrations. It has been found that 62 % of the bacteria exhibited resistance upto 1 mM, 29% have exhibited resistance up to 5 mM $HgCl_2$ and only 9% of the strains (4 isolates) have exhibited resistance upto 10 mM $HgCl_2$. The potential resistance 4 strains like AMET1217, AMET1221, AMET1229 and AMET1242 had significantly higher MICs for most of the metals as compared and these MIC results have been shown in Figure 1. The decreased growth at higher concentration of $HgCl_2$ (100µL) revealed that mercury affects the growth of bacteria. Resistance to mercury is mainly due to the physiological mechanisms of bacteria like activation, impermeability, by pass and altered target site. Early it was thought that resistance to particular substance is due to adaptation, but now it is proved that resistance is required by mutation rather than by adaptation. Metal toxicity is due to reaction of metal ions with the substance necessary for its macromolecule synthesis and finally results in the death of the organism (Summers., 1992) and this may the reason for *E. coli* sensitivity towards Hg. Resistance to mercury is not only required by transforming the plasmid from resistant to sensitive organism but also by using plasmid as a vectors, where the gene for mercury resistance is cloned and allowed to express sensitive cells[13].

**Curing of plasmids and Demonstration of plasmid mediated mercury resistance**

Among 48 strains higher metal tolerance four strains were subjected to plasmid curing experiment. It has been found that except the strain AMET1221, all other three strains have exhibited moderate mercury resistance upto the tested concentration even when they were grown in the presence of acridine orange. However, AMET1221 failed to grow in mercury amended medium after the treatment of acridine orange which clearly indicates that the strain has lost its plasmid so that only it cannot grow in the mercury amended medium and thus the plasmid mediated mercury resistance was confirmed in strain AMET1221 (Plate. 1). The resistant and sensitive bacteria were identified as respectively *Staphylococcus aureus* and *Escherichia coli.* After the transformation the plasmid DNA from *Staphylococcus aureus* in to sensitive *E. coli* which gets resistant capacity against mercury. It was screened by plating on $HgCl_2$ treated Agar plates. Transfer of resistance genes between bacteria of different species and genera occurs easily and frequently in nature[19]. Various staining and biochemical tests were performed for the identification of the selected strain AMET1221 and based on the results have that the strain AMET1221 is a *Pseudomonas* sp. and has been designated as *Pseudomonas* sp. AMET1221. Most of the strains were found to have a good degree of resistance for metals and antibiotics. It is now well-known that these properties of resistance generally reside on extra chromosomal DNA

molecule like plasmid. The genetic determinants of resistance are frequently located on plasmids or transposons[2].

**Plasmid transfer experiments**

Blue green colonies were seen in Kings B medium under UV and the growth of strain in cetrimide agar selective medium for *Pseudomonas* sp. Horizontal gene transfer of *Pseudomonas* 1221 co-cultured with *E. coli* DH5α which is chosen since it is transfer of plasmid through conjugation does not take place and does not produce endonuclease, avoid non specific digestion of plasmid DNA or permit the uptake of large plasmid (Plate. 2). The donor *Pseudomoas* sp. AMET1221 and recipient *Escherichia coli* DH5α were subjected to plasmid transfer experiments; it has been found that in control EMB agar where no heavy metal was added the number of colonies observed were 87. However, in mercury amended EMB agar, only 3 colonies were observed which clearly indicates that the rate of plasmid transfer was very less in this study. All these three colonies must have received the mercury resistant plasmid from *Pseudomoas* sp. AMET1221. In mercury amended EMB agar, only transformed *E. coli* DH5α can grow. *Pseudomoas* sp. AMET1221 cannot grow because of the presence of eosin. Untransformed *E. coli* DH5α also cannot grow because it cannot tolerate the high concentration of mercury i.e., 10 mM. The frequency of transformation was calculated to be 0.034%. It has been found that large plasmids are responsible for encoding resistance to antibiotics and heavy metals[12,2123]. Also, transferable plasmid encoding resistances to various heavy metals and antibiotics from Salmonella abortus equi[23] were reported. Contamination of environmental with toxic metals has often resulted from human activities, especially those related to mining, industrial emissions, disposal or leakage of industrial wastes, application of sewage sludge to agricultural soils, manure, fertilizer and pesticide use. Due to the potential toxicity and high persistence of metals, soils polluted with these elements are an environmental problem that requires an effective and affordable solution[9].

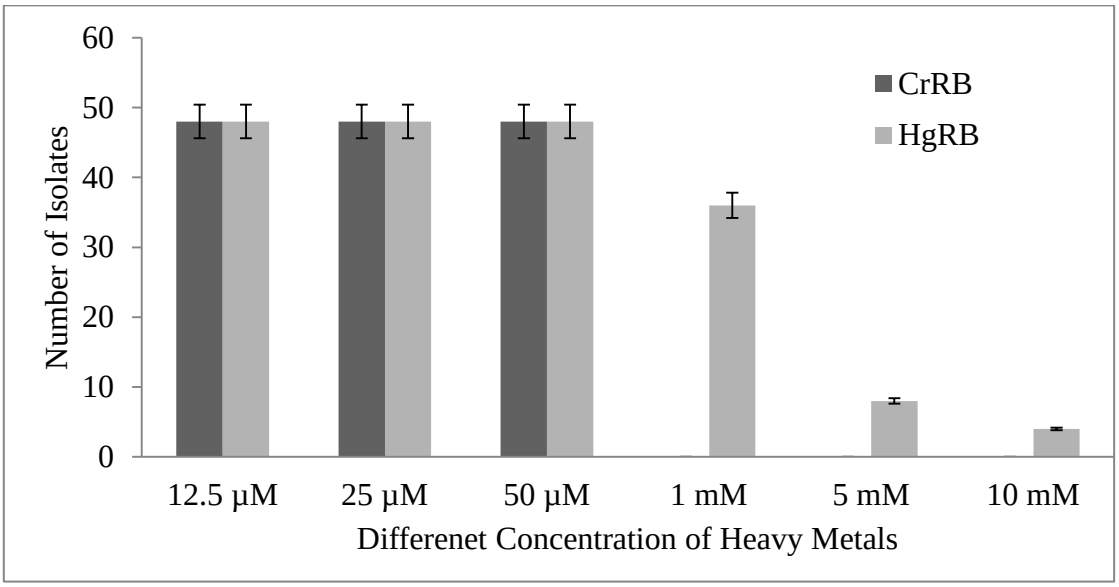


**Fig. 1: The MIC of bacterial isolates against two heavy metals. (CrRB; Chromium Resistant Bacteria, HgRB; Mercury Resistant Bacteria)**

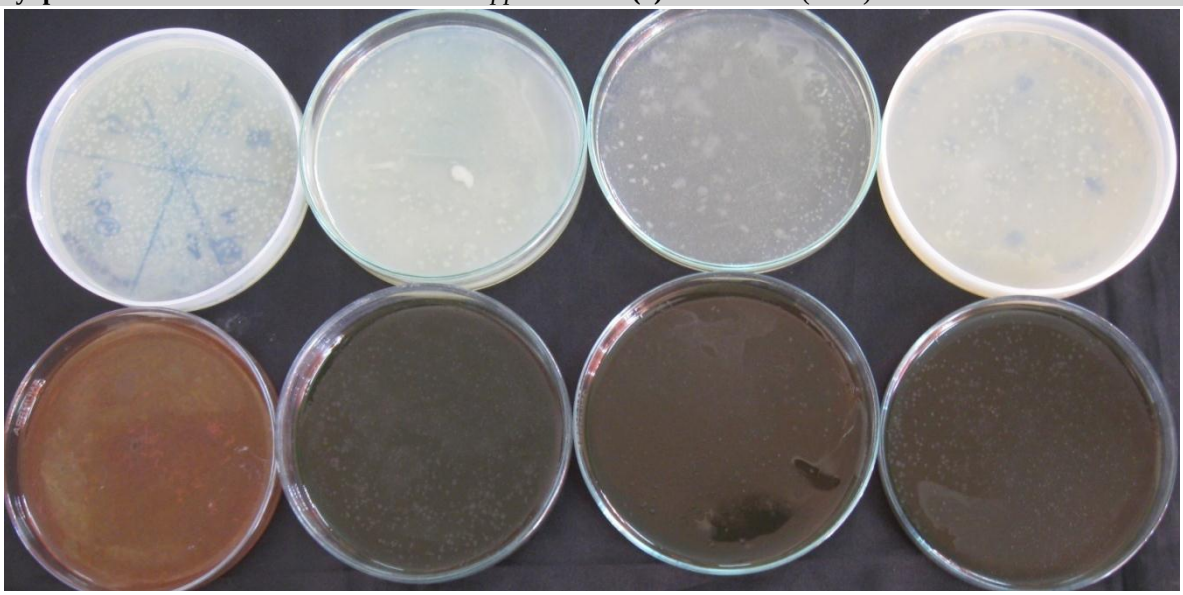

**Plate 1: Screening of transformed colonies after horizontal gene transfer**

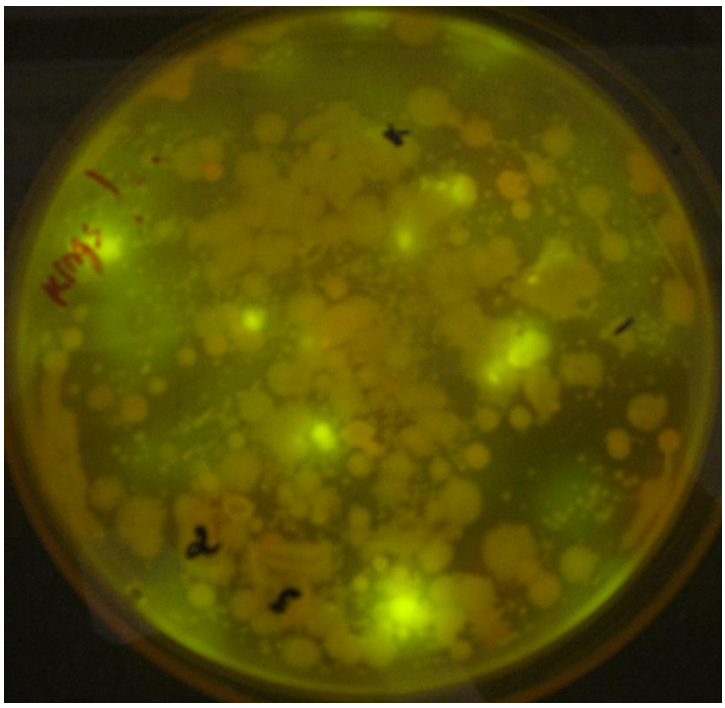

**Plate 2: Fluorescent and non fluorescent colonies on Kings B medium**

## CONCLUSION

Our study unraveled the genetic basis of the resistant of heavy metal varied between bacteria even though they isolated from the extreme environment. Experiments like plasmid curing, genetic transformations and evaluation of heavy metal resistance paved way for this confirmation. The resistance of these marine bacteria to several heavy metals enthuses to affirmatively recommend their potential to be exploited in bioremediation of heavy metals along with greater efficiency and specificity. Further investigation on marine heavy metal resistant bacteria may lead to new and better understanding of the existing concept.

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