



# Evaluation of the genetic basis of heavy metal resistance in an isolate from electronic industry effluent



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## KEYWORDS

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**Abstract** *Halomonas BVR 1* isolated from an electronic industry effluent had high level of resistance to heavy metals like cadmium, lead, zinc and to various antibiotics. Minimum Inhibitory Concentration (MIC) of the strain toward cadmium and lead was found to be 200 mg L<sup>-1</sup> and 400 mg L<sup>-1</sup> respectively, while it could tolerate zinc up to 250 mg L<sup>-1</sup> and chromium up to 150 mg L<sup>-1</sup>. The present study proved the genetic contribution of heavy metal resistance in this strain to be plasmid mediated. Isolation of the plasmid from *Halomonas BVR 1* and its subsequent linearization with *Bam HI* confirmed the presence of a plasmid of size > 10 kb. 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 *Halomonas BVR 1* with the isolated plasmid increased their metal tolerance level by 50% confirming the genetic determinant to be present in the plasmid.

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## 1. Introduction

Pollution is escalating with time due to rapid industrialization leading to an increase in various hazardous compounds liber-

ated into the environment. Environmental pollution by heavy metals from electronic industrial effluents is a global problem [13]. Heavy metals are the main group of inorganic pollutants which are continuously accumulating in our environment [15]. This is a worldwide predicament which disturbs the environmental equilibrium by gaining entry into the ecosystem due to their small size and bioaccumulation tendency [14]. Metals in higher concentrations displace the essential nutritional minerals in the living systems and prove deleterious to them by disrupting the functioning of vital organs [11,12], making it a global environmental concern.

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Electronic effluents loaded with metals and salts harbor microorganisms that have an inherent resistance to survive in such an adverse habitat. These bacterial strains may contain genetic determinants contributing to the resistance against heavy metals and are often classified to be found on plasmids, transposons or chromosomal DNA [4,5]. Recently, these innate resistance mechanisms that contribute to enhanced metal adsorption capacity by microorganisms have been exploited to carry out bioremediation. Our earlier papers have reported one such strain, *Halomonas BVR 1* isolated from a heavy metal rich electronic industry effluent and it has been clearly manifested that it is efficient in remediation of metals like lead, cadmium and zinc [7,8].

Resistance in bacteria against these toxic metals reflects the threshold of environmental contamination and the direct or indirect exposure of these bacteria to the toxic compounds. This adaptation to the adverse habitat can be natural or acquired through plasmids and the incidence of the plasmid bearing metal tolerant strains is more in a polluted site rather than an unpolluted site [6].

Organisms characterized are of interest for biotechnological purposes mainly due to production of exopolysaccharides along with their metal and antibiotic resistance. Some halotolerant bacteria are important candidates in the field of metal remediation, owing to their exceptional properties like high number of anionic and cationic functional groups on their cell surface that make them reliable for metal adsorption and intracellular accumulation processes [3].

*Halomonas BVR 1* is a novel metallophilic bacteria isolated from electronic industry effluent which grows best at an optimum salt concentration of 3–15%. Several plasmids have been identified in halophilic bacteria specifically *Halomonas* genus which confer resistance to various heavy metals. However, the genetic basis of metal resistance in our isolated strain has not been elucidated. Hence, it would be interesting to understand if the inherent metal resistance is plasmid or chromosomal mediated? Our study aims to delineate the plasmid mediated heavy metal resistance in *Halomonas BVR 1* and evaluate the increase in metal tolerance levels in non metal resistant strains by transformation of the plasmid. The plasmid mediated metal resistance found in our strain was proved by experiments like plasmid curing and genetic transformations.

Existing methods for metal remediation have certain limitations that hinder their use [3]. Hence, there is an imperative need to explore new and effective techniques for their removal. Genetic engineering technology is an alternate method to develop novel biosorbents that has the potential to improve or redesign microorganisms pertaining to their selectivity and accumulating properties of the organisms [3]. The plasmids from these metal tolerant strains may therefore be used for the genetic transformation of the lower resistant strain to boost up its metal resistance. Data from this study, can be exploited to genetically transform lower metal resistant strains contributing novel organisms to the microbial culture collection.

## 2. Materials and methods

All the experiments were carried out after approval from the Institutional Biosafety Committee (IBSC).

### 2.1. Sampling site, characterization and selection of the organism

The electronic industry effluent was characterized to identify the microbial population. The detailed procedure and the outcome of this characterization have already been reported in our earlier publication [7]. Out of the ten strains isolated, three strains belonged to the genus *Halomonas*. The organisms belonging to this genus has not been exploited as a potential biosorbent for metal remediation. Among the three strains belonging to *Halomonas* genus, *Halomonas BVR 1* was selected for further studies owing to its high tolerance level to metals and antibiotics [7].

### 2.2. Determination of the Minimum Inhibitory Concentrations (MIC)

The Minimum Inhibitory Concentration (MIC) of this selected microbe was tested against various heavy metals like cadmium, lead and Zinc. A fixed inoculum volume of 10  $\mu\text{l}$  ( $1.3 \times 10^{-7}$  cells) was inoculated into Luria Bertani medium with varied concentrations of heavy metals. Analytical grade heavy metal salts ( $\text{CdCl}_2 \cdot 8\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Pb}(\text{NO}_3)_2$ ) were used to prepare 1000  $\text{mg L}^{-1}$  stock solutions. Each of these solutions were autoclaved separately and added to LB medium at a concentration of 50–400  $\text{mg L}^{-1}$ . The growth of *Halomonas BVR 1* in this medium was analyzed by measuring the Optical Density (OD) at 600 nm [2]. This species was found to be highly resistant to cadmium with a minimal inhibitory concentration of 200  $\text{mg L}^{-1}$ . The detailed MIC of *Halomonas BVR 1* against cadmium has already been reported earlier [7].

### 2.3. Plasmid DNA isolation and digestion

Plasmid DNA was isolated using the standard alkaline lysis method proposed by Sambrook [9]. The isolated product was detected by an agarose gel (0.8%) run. The product was visualized and compared with a standard 1 kb ladder. Single digestion of the plasmid was carried out using *BamHI* with the conditions recommended by the manufacturer (New England Biolabs). The digested products were separated using 0.8% agarose gel electrophoresis. A 10 kb standard DNA ladder was run along with the digested product to assess the size of the isolated plasmid.

### 2.4. Plasmid curing experiments

To determine if the heavy metal resistance genes are encoded by the plasmid, plasmid curing experiments were carried out with ethidium bromide as the curing agent. The colonies from the highest concentration of ethidium bromide (100  $\mu\text{g/ml}$ ) were selected for testing its plasmid curing efficiency. Appropriate dilutions of the inoculum were plated onto LB agar plates. Colonies from this master plate were picked up by the process of replica plating. Accordingly, a sterilized whatman filter paper was placed upon the master plate and subsequently transferred to a selective medium of LB with optimal concentration (200  $\text{mg L}^{-1}$ ) of heavy metal lead (secondary plate). The master plate and secondary plate were compared to assess the plasmid curing efficiency. Isolation of plasmid

from the cured strain of *Halomonas BVR 1* from the master plate and non cured strains from the secondary plate was carried out to confirm the success of the curing experiments [10].

### 2.5. Genetic transformations of the isolated plasmid

Plasmid DNA obtained from a 10 ml culture in an exponential phase was eluted in a 30  $\mu$ l TE buffer (10 mM-Tris/HCl/1 mM-EDTA, pH 8). Approximately, 50 ng of this isolated plasmid was used to transform 100  $\mu$ l of *Escherichia coli* DH5 $\alpha$  competent cells [6]. Similar procedure was followed for cloning the plasmid into the cured strains of *Halomonas BVR 1* strain. This was done to know if the resistance was gained from the plasmid in the control and cured strains by this cloning experiment. These transformed strains were tested for their uptake of plasmid after transformation and increased threshold of heavy metal tolerance.

### 2.6. Assessment of the heavy metal tolerance

The transformed and non cured strains of *Halomonas BVR 1* were grown in LB medium with different lead concentrations ranging from 100 mg L<sup>-1</sup> to 400 mg L<sup>-1</sup>. Bacterial growth was measured by taking their Optical density (O.D) at 600 nm. One non metal resistant *E. coli* DH5 $\alpha$ , was taken as a control strain to compare the metal tolerance level of *Halomonas BVR 1*.

### 2.7. Decontamination of the genetically transformed strains

All the strains that were genetically modified have been discarded with utmost care. Proper decontamination of these cultures was carried out. The genetically modified organisms were developed only for the experimental purpose.

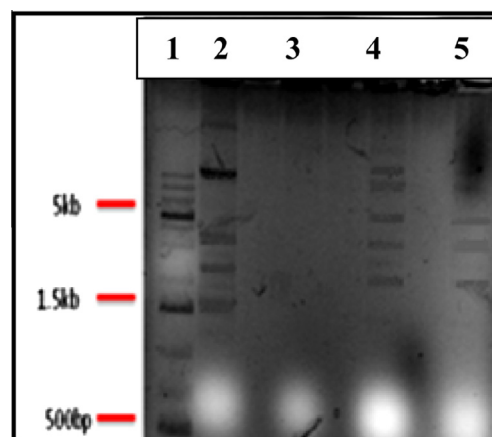
## 3. Results and discussion

Our isolated *Halomonas BVR 1*, was observed to have more resistance toward lead in comparison to cadmium and Zinc. It could tolerate lead till 400 mg L<sup>-1</sup> levels while the concentration of cadmium and zinc that inhibited the growth of the organism was 200 mg L<sup>-1</sup> and 150 mg L<sup>-1</sup> of cadmium as shown in the graph. Beyond these concentrations there was total cessation of bacterial growth. Considering these tolerance levels to various metals, this strain was selected as a test model for evaluating the genetic basis for the lead resistance in this strain.

Are genetic determinants present on plasmid?

The presence of plasmid mediated metal resistance in *Halomonas BVR 1* was substantiated by knocking out the plasmids using a plasmid curing agent. Replica plating of the cured strains on to a selective medium (LB medium supplemented with lead) reduced the growth by 50%. This is also reflected in the plasmid curing efficiency that was calculated to be around 50%. This experiment thus delineates the proof of plasmid mediated heavy metal in *Halomonas BVR 1*.

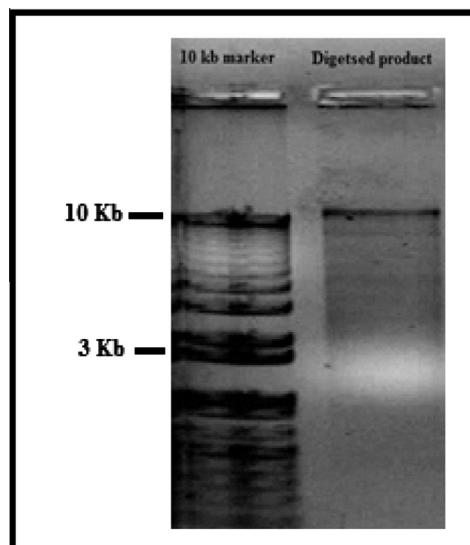
Plasmid isolation from non cured/wild type strains, cured strains and transformed strains confirmed the success of the plasmid curing experiments (Fig. 1). The agarose gel image depicted the presence of plasmid in the wild type strain while the cured strains were devoid of any plasmid. The gel image



**Figure 1** Plasmids isolated from various strains (Lane 1: Genei Step Up 1Kb marker, Lane 2: Plasmid isolated from *Halomonas BVR 1*, Lane 3: Plasmid cured from *Halomonas BVR 1*, Lane 4: Plasmid isolated from a transformed strain of *E. coli* with the plasmid of *Halomonas BVR 1*, Lane 5: Non-cured strain of *Halomonas BVR 1*).

manifested that the plasmid had multiple bands indicative of different conformations of the plasmid involving multimer, nicked circular, linear and super coiled forms. The single cut restriction digestion of this plasmid with *Bam* HI yielded a linear plasmid of size > 10 kbp (Fig. 2). The sequencing and other characterization of the plasmid are in progress in our lab.

Subsequent to genetic transformation, several individual colonies appeared on the transformed plate consisting of LB medium supplemented with lead, while there was no growth in case of competent cells. Additionally, there was one more observation made in terms of the color change in bacteria in presence of the heavy metal (Fig. 3). There was an increase in brown pigmentation observed at 400 ppm metal concentration. Bacterial pigments are known to protect the cell against



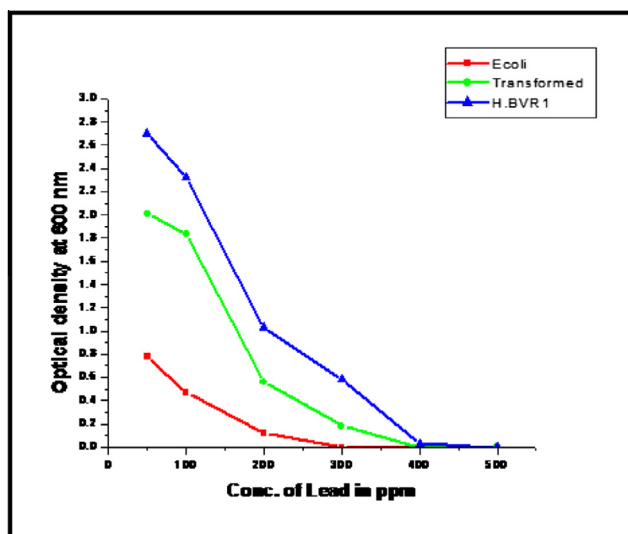
**Figure 2** Restriction digestion of the isolated plasmid from *Halomonas BVR 1* (Lane 1: NEB 10KB marker, Lane 2: Digested product).



**Figure 3** Transformed bacterial growth in the presence of heavy metal.

any photo oxidative damage caused due to the toxic metal ions. It is already reported that at low metal concentrations, bacterial pigmentation is inhibited [1]. Isolation of plasmids from the transformed strains also showed a similar kind of plasmid pattern as the wild type *Halomonas BVR 1*. This proves that the genetic transformation of heavy metal resistant plasmid in the test strain *E. coli* was successful.

The transformants, *E. coli* DH5  $\alpha$  strain and the wild type *Halomonas BVR 1* were tested for their metal resistance. It was clearly observed that *E. coli* DH5 $\alpha$  was the least resistant to lead and could tolerate heavy metal concentration only up to 300 ppm. The wild type strain *Halomonas BVR 1* had the highest metal tolerance level and could resist the metal concentration up to 400 ppm. A positive clone of *E. coli* DH5 $\alpha$  obtained after successful transformation also was tested for its metal tolerance. The experimental results showed that transformation of heavy metal resistant plasmid in the cured strains of *Halomonas BVR 1*, restored the metal tolerance level to 400 ppm after (Fig. 4). This increase in metal resistance might be attributed to the uptake of the heavy metal resistance plasmid. Overall, the plasmid curing experiments, genetic transformations



**Figure 4** Metal evaluation of *E. coli* (test strain), *E. coli* strain transformed with the plasmid from *Halomonas BVR 1* (transformed strain) and *Halomonas BVR 1* (wild strain).

and evaluation of the metal tolerance level in different strains suggested the localization of the genetic determinant bearing the property of heavy metal resistance to be present on the plasmid.

Thus, results from this study, will aid in generating microorganism with higher metal adsorption capacity along with greater efficiency and specificity. Such strains can be exploited commercially as novel biosorbents for the bioremediation of metals.

#### 4. Conclusion

Our study unraveled the genetic basis of heavy metal resistance in one isolate of electronic industry effluent. Experiments like plasmid curing, genetic transformations and evaluation of heavy metal resistance paved way for this confirmation. Results from this study, will aid in generating microorganism with higher metal adsorption capacity along with greater efficiency and specificity.

Such strains can be exploited commercially as novel biosorbents for the bioremediation of metals.

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The authors have made the following declarations about their contributions: Designing of the experiments, analyzing data and manuscript writing: Dr. Vidya Rajesh. Execution of the experimental work, analyzing data and manuscript writing: Manasi. Analysing results, contribution of reagents and manuscript writing: Dr. N. Rajesh.

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