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Heavy Metal Resistance in Bacteria Isolated from Contaminated and Uncontaminated Soils

Bina Gupta[1],
Dr. Suman Chauhan [2]

1. **Research Scholar, Department of Botany, SunRise University, Alwar (Raj.)**
2. **Research Supervisor, Department of Botany, SunRise University, Alwar (Raj.)**

Abstract

The general tolerance levels of heterotrophic bacterial populations to one or more metals can be determined by assessing their resistance to such metals. Soil bacterial communities from one uncontaminated and one metal impacted soil were analyzed to determine their resistance to some heavy metals by plating media amended with different concentrations of the metal ions. It was found that the metal-contaminated soil communities were more resistant than the uncontaminated community. In addition, the highest minimum inhibitory concentration (MIC) of more than 1000 µg/ml was observed against Pb^{2+} in 25% and 15% of the isolates in the contaminated and uncontaminated soils, respectively. On the other hand, the MIC for Cu^{2+} and Cr^{6+} ranged between 200 and 600 µg/ml. Most bacterial isolates from the soil were resistant to very high concentrations of heavy metals regardless of the level of metal concentrations in their environment. It is proposed that the resistance ability of the isolates could be exploited in considering the isolates as possible candidates for the decontamination of metal-polluted sites. The most predominant isolates at high concentrations of the metal ions include *Bacillus* spp., *Pseudomonas* spp., *Corynebacterium* spp., *Micrococcus* spp. and *Flavobacterium* spp.

Keywords; heavy metal, resistant, pollutant, detoxification, contamination.

Introduction

Heavy metals are considered serious pollutants because of their toxicity, persistent and nondegradable conditions in the environment (Tam and Wong, 2000; Yuan *et al.*, 2004). In India, soil pollution problems associated with spilling of automobile wastes has been reported (Ipeaiyeda *et al.*, 2008; Iwegbue, 2007). Such spilling of automobile wastes arises from human activities in mechanic villages. A mechanic village is an area of open land usually allocated to automobile repair workers in the vicinity of an urban centre. A typical city usually has one or more of the mechanic villages, in proportion to its population and activities. Several reports have shown the existence of anthropogenic dispersion and concentration of heavy metals in soil (Remon *et al.*, 2005; Liu *et al.*, 2007; Onweremadu and Duruigbo, 2007).

Studies have shown that long-term heavy metal contamination of soils has harmful effects on soil microbial activity, especially microbial respiration (Doelman and Haanstra, 1979). Aside from long-term metal-mediated changes in soil enzyme activities, many reports have shown large reductions in microbial activity due to short-term exposure to toxic metals (Doelman and Haanstra, 1984). Bacterial activity, measured by thymidine incorporation technique, had been shown to be very sensitive to metal pollution (Diaz-Ravina and Baath, 1996a, b). Moreover, habitats that have high levels of metal contamination show lower numbers of microbes than uncontaminated habitats (Kandeler *et al*., 2000).

In soil, the interplay of microbial metal mobilizing mechanisms and metal fixation forces is highly complex and dependent on a number of soil characteristics (Haferburg and Kothe, 2007). Metals without biological function are, in general, tolerated in minute concentrations, whereas

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essential metals with biological functions are usually tolerated at higher concentrations. They accomplish either metabolic functions as constituents of enzymes or meet structural demands as, e.g., in supporting the cell envelope. Depending on the external conditions, microbial cells have developed mechanisms to cope with high concentrations of metals (Silver and Misra, 1988).

Heavy metals affect microbial cells in various ways. It has been shown that the impact of metals on microbial metabolism is dependent on the growth form, while in consortia from mining sites, the resistance towards different metals seems to be higher than for pure cultures (Sprocati *et al*., 2006).

A great number of heavy metal resistant bacteria is known to possess efflux transporters that excrete toxic or overconcentrated metals (Nies, 2003). Many bacteria isolated from soil have been found to be resistant to very high concentrations of heavy metals (Angle *et al*., 1993).

The ability of microorganisms to survive toxic effects of heavy metal exposure is due to some intrinsic property and detoxification mechanisms and other resistant mechanisms (Winge *et al*., 1989). The environmental effects of heavy metals could be assessed by determining the number of metal – resistant bacteria isolated from an environment affected by heavy metals. Theoretically, if a significant proportion of the bacterial population is resistant to high concentrations of the metal contaminant, then the judgement is made that the soil is negatively affected by the presence of the metal (Olson and Thornton, 1981).

Different organisms exhibit diverse responses to toxic ions, which confer upon them a certain range of metal tolerance (Valls and de Lorenzo, 2002). Bacteria thus show a panoply of responses to metal ions and diverse bacterial groups have developed abilities to cope with these toxic elements in a variety of environments. With respect to pollution control, these activities

show promise with regard to mobilization of metals, designing of metal-tolerant strains and metal bioremediation through the breeding of natural or engineered strains (Gadd, 2000). It has been stated by Leduc *et al.* (1997) that for a biohydrometallurgical process to be effective, the bacterium used must be resistant to the metal recovered as well as to others in the environment. The phenomenon of microbial resistance is of some fundamental importance and is particularly relevant in microbial ecology, especially in connection with the roles of microbes in polluted ecosystems and in the reclamation of metal-contaminated natural habitats. Microbes can be used in locating ore deposits by searching for bacteria with unusually high resistance to the metal constituent(s) of the ore sought (Ehrlich, 1992).

Microbial populations in metal-polluted environments contain organisms often described as metal-tolerant (Duxbury and Bicknell, 1983). It has been shown by Timoney *et al*. (1978) that mercury-resistant bacteria were only isolated in substantial numbers from mercury-polluted sediments. Angle *et al*. (1993) reported that most bacteria isolated from soil were resistant to very high concentrations of heavy metals, regardless of whether or not the soils were contaminated with metals.

Waste dump sites in mechanic villages and elsewhere have been reported to be contaminated with heavy metals (Liu *et al*., 2007; Nwachukwu *et al*., 2010; Odukoya and Abimbola, 2010) are likely sources of heavy metal resistant microorganisms. This study aimed to isolate, identify and compare heavy metal resistant bacteria from contaminated and uncontaminated soils in order to assess whether resistance was associated with presence of metal pollution.

Materials and methods

Collection of samples

Two soils were used for the study. One of the soils, designated A and referred to as the metal-contaminated soil, was collected from a metal-wastes dump site in the mechanic village in Nsukka town which has been used for such for over fifteen years. The other soil, B, with no known history of metal contamination was collected from the zoological garden of the Sunrise University, Alwar, and referred to as the uncontaminated soil. Three different points were randomly selected at each site from where the soil samples were collected. The soil samples were aseptically collected from a depth of 0 -15 cm and put into sterile screw-cap containers and transported to the laboratory immediately for analyses.

Preparation of soil samples for analyses

All the three soil samples from each location were lumped together, thoroughly homogenized, air-dried and sieved with a 2 mm wire mesh before analyses.

Determination of some properties and total metal concentration of soil

Particle size analyses of the soil samples were determined by the hydrometer method of Bouyoucos (1951). The heavy metal analysis of the soil samples was done by the method of Alef and Nannipieri (1985) after which the total metal concentration was determined by atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk).

Isolation of metal-resistant bacteria

The selective isolation of soil bacterial populations resistant to some heavy metal ions was carried out by surface-plating dilutions of soil suspension onto nutrient agar supplemented with different concentrations of different metal salts. One gramme (wet weight) of each soil sample was suspended in 9.0 ml sterile distilled water, serially diluted in ten-fold before plating out.

Aliquots (0.1ml) of the diluted samples were spread over the surface of nutrient agar plates amended with 10, 100, 200, 500 and 1000 µg/ml of $Pb(NO_3)_2$, $Cu(NO_3)_2$ and $K_2Cr_2O_7$. Control plates, without added metal salts, were also inoculated. All plates, in duplicates, were incubated at 30°C for 10 days, after which total counts were taken.

Predominant colonies of different morphological types were selected from each of the inoculated plates at the highest concentration which showed growth for both soils and for each metal. The selected isolates were subcultured repeatedly on nutrient agar plates containing the same metal salt concentration as in their isolation medium. Morphological dissimilarity was used as a criterion for isolate selection because when soil populations were plated onto metal-amended media, population diversity decreased and colonies of the same morphology were represented several times on the same plate (Duxbury and Bicknell, 1983). Three serial streakings of each culture were done to ensure purity of the strains.

Determination of minimum inhibitory concentration (MIC) of heavy metals

To evaluate the level of metal resistance of the soil bacterial community, the MIC of individual bacterial strains randomly selected from the soil isolates was determined. The soil samples were serially diluted and appropriate aliquots plated onto nutrient agar medium. No metal selection was used during the isolation. Twenty randomly selected isolates were collected from each soil and purified by repeated streaking onto nutrient agar medium. Each isolate was then streaked onto media containing a range of concentrations of each metal salt. Stock solutions of the metal salts were prepared in sterile distilled water and added to the medium to achieve the desired concentrations ranging from 10 to 1000 µg/ml which were used. Each selected isolate was grown overnight in nutrient broth at 30°C and streaked on the metal-amended medium. All

the plates were incubated at 30°C for 24 to 72 h. The lowest metal salt concentration that inhibited growth of each isolate was taken as the MIC of the metal against the isolate tested.

Characterization and identification of isolates

All the predominant bacterial isolates obtained at very high metal concentrations in the metal resistance study were purified, characterized and identified on the basis of their cultural characteristics, morphology, motility and biochemical reactions based on the schemes of Barrow and Feltham (1993) and Holt *et al*. (1994).

Statistical analysis.

Results were evaluated with Student's two-tailed t test and one-way analysis of variance, using Tukey's HSD procedure (Steel and Torrie, 1980). The level of significance was set at $P < 0.05$ for all comparisons.

Results

The two soils used in the investigation are both sandy loam based on their particle size distribution. Some of the properties of the soil samples are shown in Table 1. Analysis of the soil samples for total metal concentrations showed that there were more elevated levels of the metals in soil A than soil B. The total concentration of Cr in soil A was 353.2 µg/g, a three-fold value higher than that of soil B which was 112.4 µg/g. The concentration of Cu was found to be 261.2 µg/g for soil A and 108.5 µg/g for soil B, while for Pb, it was 223.3 µg/g for soil A and 84.6 µg/g, over two-and-half fold value higher than that for soil B (Table 1).

According to the results obtained in this study, there was no significant difference ($P< 0.05$) in the total bacterial counts of soils A and B as there were about as many bacteria in both

unamended soils. This is shown in Table 2. Thus, the higher level of total metal concentrations in soil A than soil B does not translate, proportionately, to differences in microbial load.

The addition of either low or high levels of chromium resulted in extended lag periods of growth beyond those for treatments without chromium or those to which copper or lead was added. This showed more toxicity by chromium than lead and copper. Moreover chromium, at 500 µg/ml inhibited bacterial growth in soil B while at 1000 µg/ml, growth was inhibited in both soils A and B.

Media amended with lead salt showed the greatest abundance of growth when compared with media amended with copper and chromium at all concentrations indicating more resistance to lead by the soil bacterial community than to chromium and copper. The growth of bacteria in the presence of lead was significantly different as compared with copper and chromium, concentration by concentration. When media were amended with 10 µg/ml of metal salt, there was no significant difference in total counts with the control soils for lead and copper (Table 2). However, significant changes in the number of bacterial colonies were obtained when the media were supplemented with concentrations of metal salts above the 10 µg/ml level. Comparing the media amended with Pb at 100 µg/ml to control media, bacterial counts obtained from soils A and B decreased by one order of magnitude, and by two orders of magnitude for 200 µg/ml. For the 500 and 1000 µg/ml concentrations of lead, up to four and six orders of magnitude decreases were, respectively, observed in counts from both soils.

For soils A and B amended with copper and chromium salts at 100 µg/ml concentrations and above, there were significant differences ($P < 0.05$) for corresponding metal concentrations. Both soils gave bacterial counts that decreased by different orders of magnitude as the metal

concentration increased. However, at 500 µg/ml, there was a decrease of six orders of magnitude for bacterial counts in soil A without any growth observed in soil B.

These results suggest some level of toxicity of the metal salts used whereas resistant strains of bacteria still emerged. There were decreases in population diversity with increasing metal concentrations. The predominant bacterial strains isolated from the 1000 µg/ml lead – amended media belonged to *Bacillus* spp, *Pseudomonas* spp. *Corynebacterium* spp and *Micrococcus* spp. In the copper-amended media at 500 µg ml^{-1}, the predominant isolates were *Flavobacterium* spp., *Bacillus* spp., *Corynebacterium* spp., and *Pseudomonas* spp. while in the chromium-amended media at 500 µg ml^{-1}, *Thiobacillus* spp., *Bacillus* spp, *Acinetobacter* spp., *Corynebacterium* spp. and *Pseudomonas* spp. were the predominant strains isolated. These strains exhibited resistance to the metals at the stated concentrations.

The twenty randomly selected isolates were tested for their level of resistance against the three heavy metal ions, namely, Cr^{6+}, Cu^{2+} and Pb^{2+}. The MIC of the randomly selected isolates showed high levels of resistance against the heavy metal ions. There was significant difference in the resistance levels of the bacterial community of soils A and B as the isolates obtained from soil A showed higher levels of resistance than isolates from soil B (Table 3). It was observed that most isolates were resistant to metal concentrations much higher than the total metal concentrations found in any of the soils (Table 3). In soil sample A, the MIC of >1000 µg ml^{-1} was observed against Pb^{2+} in 25% of the isolates while it was 15% for soil B. On the other hand, the isolates were resistant to Cu^{2+} and Cr^{6+} ions to lesser degrees (Table 3). The isolates originally obtained from the soils on nutrient agar medium possess an intrinsic degree of metal tolerance that generally precludes their need to adapt to the introduction of metal stress. There

was no general correlation between the total metal content of the soils and the percentage of bacteria resistant to the particular metal.

In general, the media amended with lead supported the largest number of resistant strains followed by copper and then chromium. Thus Cr^{6+} ion appeared the most toxic ion followed by Cu^{2+} and then Pb^{2+}.

Discussion

A wide range of microorganisms from all the major groups of bacteria could be found in metal-polluted habitats as are in non-polluted sites. In the polluted site, bacteria are continuously exposed to different heavy metals, thus giving rise to survival of metal tolerant strains. Even some of the strains which were not metal tolerant may become tolerant, possibly, due to mutations. Thus these strains assist in natural transformation leading to increased incidence of metal tolerant strains in such environment and also dissemination to atmosphere.

In this study, increase in concentrations of the metal ions caused corresponding decrease in soil microbial population and diversity. Comparative studies by Fliessbaach *et al.* (1994) and McGrath *et al.* (1995) have shown reductions in microbial biomass or soil enzyme activities for agricultural soils amended with metal-containing sewage sludge. Kuperman and Carreiro (1997) have shown that heavy metal contamination of soil adversely affects the abundance and activity of microorganisms involved in organic matter decomposition and nutrient cycling. However, metal-tolerant bacteria can account for a sizeable proportion of the total heterotrophic bacterial populations suggesting the evolution of metal-ion resistant strains.

The metal resistant test showed that some of the selected isolates had MIC of over 1000 µg/ml against lead. The MIC for Cu^{2+} and Cr^{6+} ranged between 200 and 600 µg/ml. Rajbanshi

(2008) had reported MIC of 150 to 500 µg/ml for chromium and 200 to 300 µg/ml for copper in different bacteria. Brocklehrust and Morby (2000) reported that in response to toxic concentrations of heavy metal ions, *E. coli* strains exhibited varying degrees of tolerance (3 -14-fold) both to the adaptive metal and its congeners. The ability of microorganisms to grow in the presence of relatively high metal ion concentrations is found in a wide range of microbial groups and species, including those from unpolluted sites and not in all cases is any adaptation necessary (Gadd, 1990). The adaptation to heavy metal rich environments is resulting in microorganisms which show activities for biosorption, bioprecipitation, extracellular sequestration, transport mechanisms, and/or chelation (Haferburg and Kothe, 2007). Such resistance mechanisms are the basis for the use of microorganisms in bioremediation approaches.

The effects of metal ion stress on microbial cells/communities suggest that individual strains adapt to elevated metal-ion concentrations (Giller *et al*., 1998). Exposure to heavy metals selects for resistance to heavy metals in the surviving microorganisms (Coyne, 1999). However, prior exposure to one heavy metal does not mean better survival when a different heavy metal is present. Microorganisms may persist in soils contaminated with extremely high heavy metal concentrations because those heavy metals may be extractable but not biologically available

In the present study, *Bacillus* spp and *Corynebacterium* spp. were encountered as resistant to copper and lead. Such characteristic has been reported by Sharma and Thapaliya (2009).

Bacterial resistance to heavy metals may be a fallout of the detoxification mechanisms intrinsic to the bacteria. Such processes have potential for application in bioremediation. The removal of pollutants, such as heavy metal compounds, metalloids, radionuclides, organometal(loid)s and related substances, from contaminated sites by living or dead microbial

biomass or their products may provide an economically feasible and technologically efficient means for element recovery and environmental protection.

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Table 1. Some properties and total metal concentrations of soil samples.

Characteristic	Soil A	Soil B
Sand	74%	68%
Silt	15%	20%
Clay	11%	12%
Organic carbon	4.12%	3.82%
Organic matter	7.09%	6.57%
Total nitrogen	0.46%	0.52%
Cation exchange capacity	165 mmol/kg	160 mmol/kg
pH	6.38	6.56
Chromium	353.2 µg g^{-1}	112.4 µg g^{-1}
Copper	261.2 µg g^{-1}	108.5 µg g^{-1}
Lead	223.3 µg g^{-1}	84.6 µg g^{-1}

Table 2: Total number of heterotrophic bacteria resistant to concentrations of metal salts.

Metal salt in medium (µg/ml)	Soil type A	Soil type B
	Total bacterial population in soil	
$Pb(NO_3)_2$		
0	3.8×10^8	4.1×10^8
10	3.0×10^8	2.8×10^8
100	5.6×10^7	3.2×10^7
200	3.3×10^6	2.0×10^6
500	6.4×10^4	4.3×10^4
1000	2.7×10^2	1.6×10^2
$Cu(NO_3)_2$		
0	3.8×10^8	4.1×10^8
10	3.2×10^8	3.0×10^8
100	4.3×10^6	2.2×10^6
200	6.2×10^4	8.6×10^3
500	3.8×10^2	n
1000	n	n
$K_2Cr_2O_7$		
0	3.8×10^8	4.1×10^8
10	1.7×10^8	1.5×10^8
100	2.1×10^6	7.6×10^5
200	3.8×10^4	3.1×10^2
500	1.2×10^2	n
1000	n	n

n – no growth

Table 3: Minimum inhibitory concentration of heavy metals against selected isolates.

Minimum inhibitory concentration (µg/ml)

Isolate	Soil A			Soil B		
	Pb	Cu	Cr	Pb	Cu	Cr
1	600	400	250	800	450	400
2	500	400	300	500	350	200
3	600	350	200	600	450	300
4	450	300	200	500	300	200
5	>1000	500	350	600	200	450
6	400	250	200	>1000	350	400
7	350	450	300	500	350	250
8	500	350	300	>1000	450	400
9	>1000	450	500	500	400	350
10	500	450	400	850	400	450
11	800	500	500	600	450	350
12	>1000	600	350	600	450	400
13	500	450	450	500	450	300
14	400	400	350	600	300	400
15	>1000	600	450	400	400	300
16	600	450	500	500	350	400
17	500	500	400	>1000	400	400
18	600	500	400	500	400	400
19	>1000	600	450	600	450	350
20	500	400	250	500	400	400