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Original Research Paper

A Study on Antibiotic Resistance and Metal Tolerance of Bacteria Isolated from Industrial Site

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ABSTRACT

The objective of the present study was to screen for antibiotic resistance and metal tolerant bacteria. Soil samples were collected from industrial contaminated sites for antibiotic resistance and metal tolerance. As the industrial effluents contain high concentration of heavy metals along with other organic and inorganic pollutants it was chosen for the study. Eight distinct bacterial species were isolated and tested for the metal tolerance (Cr, Ni and Mn) and antibiotic resistance (bacitracin, chloramphenicol, streptomycin, rifampicin, penicillin and ampicillin) at minimum inhibitory concentration (MIC). It was found that most of the isolates had multiple antibiotic resistance which may be due to heavy metals released in industrial sites. The multiple antibiotic resistance of bacterial species was also associated with tolerance to metals such as chromium, nickel and manganese.

INTRODUCTION

Bacterial resistance to antibiotics and other antimicrobial agents is an increasing problem in today's society. Anthropogenic activities such as mining operations and discharges of industrial wastes, have resulted in accumulation of metals in the environment and the food chains leading to serious ecological and health problems (Hong et al. 1996**)**. Antibiotics and metal resistant microorganisms have been isolated from nosocomial and burn wound infections, infections treated with metal based antimicrobial agents and various metal contaminated environments such as estuaries, soils and sewage (Calomiris et al. 1984). Distillery effluent site contains heavy metals and other organic and inorganic pollutants. The work on distillery heavy metals shows highly resistant strains of bacteria to both metals as well as antibiotics (Asthana et al. 2004). Antibiotic resistance genes in most bacteria are frequently found in extra chromosomal elements known as R plasmids (Carrasco et al. 1997). Although some heavy metals are essential as trace elements, but at high concentrations they become toxic to all life, including microbes, by forming complex compounds within the cell. Microbes have evolved several mechanisms to tolerate the presence of heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of metal ions inside the cell and reduction of the heavy metal ions to a less toxic state. Because the intake and subsequent efflux of heavy metal ions by microbes usually include a redox reaction involving the metal, bacteria that are resistant to and grow on metals also play an important role in the biogeochemical cycling of those metal ions (Adarsh et al. 2007). Heavy metal resistance in bacteria has been shown to be associated with single or multiple drug resistance. *Staphylococcus aureus* strain has clearly demonstrated a correlation between resistance to penicillin, erythromycin, and tetracycline and tolerance to mercury, lead, cadmium and zinc. Similar resistance patterns exist for strains of *Escherichia coli, Salmonella typhimurium,* and *Pseudomonas aeruginosa* (Allen et al. 1977). Some bacteria have evolved

mechanisms to detoxify heavy metals, and some even use them for respiration. Because the intake and subsequent efflux of heavy metal ions by microbes usually include a redox reaction involving the metal (that some bacteria can even use for energy and growth), bacteria that are resistant to and grow on metals also play an important role in the biogeochemical cycling of those metal ions. This is an important implication of microbial heavy metal tolerance because the oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself (Anne Spain & Elizabeth Alm 2003). The main objectives of this study were to isolate the bacterial species and test them for their antibiotic resistance and metal tolerance along with metal uptake capacity.

MATERIALS AND METHODS

Sample collection and isolation of microorganisms: Bacteria were isolated from industrial soil sample. Screening was conducted to isolate antibiotic resistant strains and metal tolerant organisms. Samples were collected from different industrial effluent sites in and around Vellore district in India. For isolation and enumeration of microorganisms, samples were serially diluted in sterile distilled water and plated on nutrient agar plates, which were incubated at 37°C for 48 h, and screening of colonies was performed.

Identification and characterization of the bacterial isolates: The shape and colour of the colonies were examined under the microscope after Gram's staining. Motility test was also done. Isolates were biochemically analysed for the activities of oxidase, catalase, MR-VP test, starch hydrolysis and gelatin hydrolysis, indole production, hydrogen sulphide test, nitrate reduction, urease and citrate utilization. The results were compared with Bergey's Manual of Systematic Bacteriology.

Study of antibiotic resistance: All isolates were separately tested for their resistance to antibiotics like bacitracin, chloramphenicol, streptomycin, rifampicin, penicillin and ampicillin at their minimum inhibitory concentration (MIC) on Muller Hinton agar by disc diffusion method. The antibiotic disc was placed on the surface of the culture plates by using forceps dipped in alcohol and flamed, each disc was gently pressed down with sterile forceps to ensure that disc adheres to the surface of the agar. All plates were incubated at 37°C for 18 to 24 h. After incubation, the diameter of the inhibition zones around the discs was measured. The antibiotic disc and their concentrations used were bacitracin (8 units/disc), chloramphenicol (10 mcg/disc), streptomycin (10 mcg/disc), rifampicin (15 mcg/disc), penicillin (10 units/disc) and ampicillin (10 mcg/disc) respectively.

Metal tolerance assay: All isolates were separately tested for their tolerance to three metals (chromium, nickel and manganese) by well diffusion method. The compounds used were $K_2Cr_2O_7$, $NiCl_2.6H_2O$ and $MnSO_4$ respectively. Totally five concentrations i.e., 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm of metal stock solutions were prepared and then transferred to wells in the Muller Hinton agar plates. The initial concentration used (100 ppm) was added from 100 mg/100 mL stock solution. The stock solutions of $K_2Cr_2O_7$, $NiCl_2.6H_2O$ and $MnSO_4$ were prepared in double distilled water and sterilized by autoclaving at 121°C, 15 lbs pressure for 15 min. Zone of inhibition was determined after 48 h of incubation at 37°C.

Estimation of heavy metals: The biosorption of heavy metals was carried out using bacteria grown in 250 mL conical flasks containing 50 mL of LB medium supplemented with heavy metals at the concentration of 100 mg/L (Cr, Ni and Mn) and incubated at 37°C for 48 h. After the samples were harvested by centrifugation at 5000 rev/min, supernatant was collected for heavy metal analysis. The heavy metals present in the solution were determined by Atomic Absorption Spectrophotometer.

The amount of metals in samples was estimated by using known concentrations of metals in the medium as control.

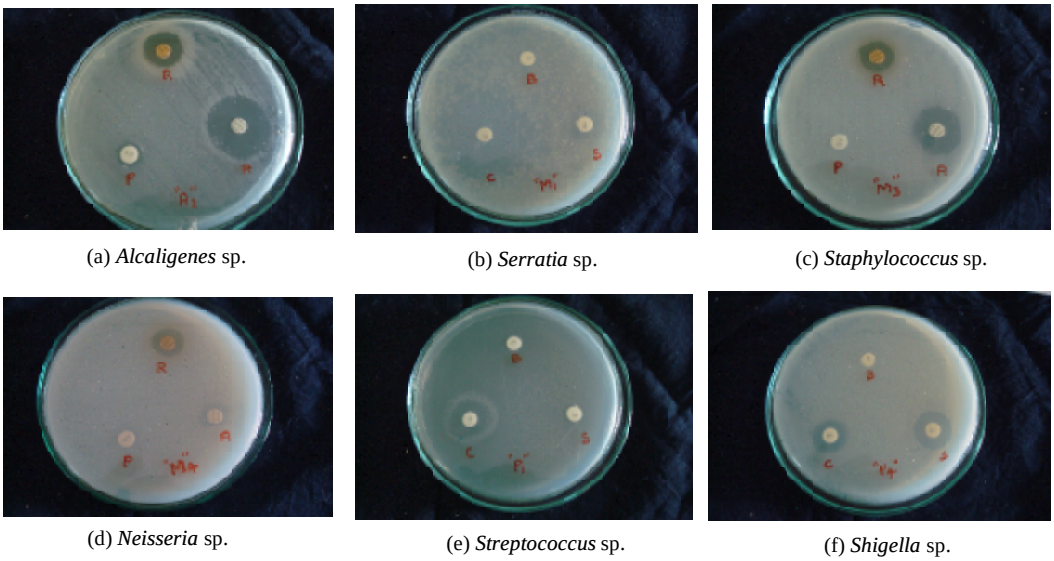
RESULTS AND DISCUSSION

Isolation of bacterial species: The eight different bacterial species were isolated from contaminated industrial soil samples. The isolated bacterial species were *Alcaligenes* sp., *Serratia* sp., *Staphylococcal* sp., *Neisseria* sp., Streptococcus sp., *Micrococcus* sp., *Shigella* sp. and *Enterobacter* sp. The biochemical characteristics of bacterial isolates are presented in Table 1.

Antibiotic resistance: Most of the isolates were multiple antibiotic resistant. *Neisseria* sp. was the only organism which was found to be resistant to all the antibiotics used in the present study. *Alcaligenes* sp. were resistant to all antibiotics expect ampicillin, while *Serratia* sp. were resistant to streptomycin and ampicillin. *Staphylococcus* sp. was sensitive to chloramphenicol and streptomycin. The species of *Streptococcus* sp and *Micrococcus* were resistant to bacitracin, streptomycin and ampicillin, and *Micrococcus* sp was also resistant to rifampicin. Both *Shigella* sp. and *Enterobacter* sp. were sensitive to one antibiotic and resistant to the rest (Table 2, Fig. 1).

Metal tolerance assay: The bacterial species showed very high degree of tolerance to the heavy metals Cr, Ni and Mn. MIC values varied from 100 to 500 ppm (Table 3). Organisms were able to tolerate nickel and manganese in all the concentrations chosen for the study. However, marked variations were noticed when they were exposed to chromium. *Alcaligenes* sp. and *Staphylococcus* sp. were found to tolerate chromium even up to 500ppm, whereas *Neisseria* sp. and *Sterptococcus* sp. could tolerate only up to 200ppm, and *Serretia* sp., *Micrococcus* sp. and *Shigella* sp. were able to withstand the initial dose of 100ppm.

Biosorption of metals: Metal uptake capacity was investigated using heavy metals at different time intervals of growth up to 48 hours. Decrease of metal concentration in solutions was observed with



(a) *Alcaligenes* sp. (b) *Serratia* sp. (c) *Staphylococcus* sp.

(d) *Neisseria* sp. (e) *Streptococcus* sp. (f) *Shigella* sp.

Fig. 1: Plate showing antibiotic resistance of bacterial isolates
B = Bacitracin, C = Chloramphenicol, S = Streptomycin, R = Rifampicin, P = Penicillin, A = Ampicillin

Table1: Biochemical characteristics of bacterial isolates from contaminated industrial site.

S. no	Biochemical tests	*Staphylo-coccus* sp	*Streptoc-occus* sp	*Micrococ-cus* sp	*Alcali-genes* sp	*Serra-tia* sp	*Neiss-eria* sp	*Shig-ella* sp	*Entero-bacter* sp
1	Indole test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	Methyl red test	+ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve
3	Voges proskauer test	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
4	Citrate utilization test	-ve	-ve	-ve	-ve	+ve weak	-ve	-ve	+ve
5	Catalase test	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
6	Oxidase test	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve
7	TSI test	NP	NP	NP	KS/NCB	AS/AB	AS/AB	KS/AB	AS/AB
8	Urease test	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
9	Nitrate reduction test	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
10	H_2S test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
11	Starch test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
12	Gelatin liquefaction test	+ve	-ve	+ve slow	-ve	+ve	+ve	-ve	-ve
13	Glucose	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
14	Sucrose	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve
15	Lactose	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve
16	Phenylalanine test	NP	NP	NP	-ve	-ve	-ve	-ve	-ve

Note: +ve = Positive, KS = Alkaline slant, NCB = No change butt, -ve = Negative, AB = Acid butt , AS = Acid slant, NP= Not Performed

Table 2: Antibiotic resistance of bacterial isolates from contaminated industrial site.

Antibiotic	B	C	S	R	P	A
Alcaligenes sp.	R	R	R	R	R	S
Serratia sp.	I	S	R	S	I	R
Staphylococcus sp.	R	S	S	R	R	R
Neisseria sp.	R	R	R	R	R	R
Streptococcus sp.	R	S	R	S	S	R
Micrococcus sp.	R	S	R	R	I	R
Shigella sp.	R	I	S	R	R	R
Enterococcus sp.	R	S	R	R	I	R

B = Bacitracin, C = Chloramphenicol, S = Streptomycin, R = Rifampicin, P = Penicillin, A = Ampicillin
R = Resistant, S = Sensitive, I = Intermediate

Table 3: Metal tolerance of bacterial isolates from contaminated industrial site.

Genus	Chromium (100 to 500 ppm)					Nickel (100 to 500 ppm)					Manganese (100 to 500 ppm)				
Alcaligenes sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Serratia sp	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Staphylococcus sp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neisseria sp	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Streptococcus sp	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Micrococcus sp	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Shigella sp	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Enterococcus sp	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+

+ = Resistant, - = Sensitive

Table 4: Heavy metal uptake of bacteria isolated from contaminated industrial site.

Bacterial Isolates	Concentration (mg/L) exposed for duration of 48 h		
	Chromium	Nickel	Manganese
Alcaligenes sp.	49.08	56.19	61.06
Serratia sp.	38.31	47.70	62.34
Staphylococcus sp.	43.71	47.04	63.32
Neisseria sp.	39.87	55.50	61.30
Streptococcus sp.	39.91	56.99	62.11
Micrococcus sp.	37.54	55.71	65.68
Shigella sp.	37.35	55.91	66.34
Enterococcus sp.	48.57	55.47	64.34

the increase in the growth due to efficient uptake of metals with a maximum time at 48 hours. The uptake capacity was measured by atomic absorption spectrophotometer (Table 4, Fig. 2). Pronounced variation in chromium uptake was noticed among the organisms. The maximum uptake was recorded by *Alcaligenes* sp., and the minimum by *Shigella* sp. and *Micrococcus* sp. The uptake of nickel and manganese was more or less similar to all the organisms.

Antibiotic resistance and metal tolerance were found in bacterial species isolated from contaminated industrial soils. The isolates were identified as *Alcaligenes* sp., *Serratia* sp., *Staphylococcus* sp., *Neisseria* sp., *Streptococcus* sp., *Micrococcus* sp., *Shigella* sp. and *Enterobacter* sp. The results were similar to the previous works reporting the presence of these bacterial isolates in metal contaminated environments (Asthana et al. 2004). An association or direct linkage between antibiotic and metal has been demonstrated (McHugh et al. 1975). Others have demonstrated genetic linkages (presumably by plasmids) between antibiotic resistance in *Enterobacter aerogenes* and tolerance to Cd and Zn (Pickett & Dean 1976). Penicillinase plasmids of *Staphylococcus aureus* have been shown responsible for resistance to erythromycin and various inorganic ions, including Cd, Pb, Hg and Zn (Novick et al. 1968). There are similar findings in our study were both *Staphylococcus* sp. and *Alcaligenes* sp. were found to be tolerant to all the three metals at 500 ppm. The prevalence of metal tolerant and antibiotic resistant microorganisms are ecologically very important as both characters are plasmid borne. Under environmental conditions of metal stress, these organisms will adopt faster multiplication and spread of R-factor than by mutation and natural selection, thus, leading to a very rapid increase in their numbers (Carrasco et al. 1997). *Pseudomonas aeruginosa* BC15 was capable of removing significant amount of Ni, Pb, Cd and Cr during growth within 48 hours even though it has been reported that the metal biosorption capacity of microbial cells varies with the growth phase (Maceskie & Dean 1984). BC15 was able to remove 65% Pb and 30% of Cr (VI) from mixed metal solutions (100mg/L) within 48 h when compared to *Staphylococcus saprophyticus*, which could remove 100% Pb, 25% Cr (VI) and 24% Cu respectively (Ilhan et al. 2004). *Enterobacter cloacae* and *Klebsiella* species have been resistant against Cd, Cr and Pb. *Enterobacter cloacae* resisted Cd (220 mg/L), Cr (800 mg/L) and Pb (1400 mg/L). *Klebsiella* strain (CMBL-Cd-2 and CMBL Cd-

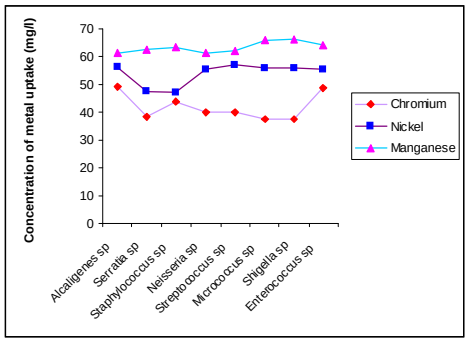


Fig. 2: Heavy metal uptake of the bacterial species isolated from soil sample.

3) showed resistance to Cd at the concentration of 110 mg/L and 100 mg/L, Cr 600 mg/L and 500 mg/L, Pb 1200 mg/L and 900 mg/L supplemented in the medium respectively (Riazul et al. 1999). Thompson & Walting (1987) reported up to 25% Pb removal using pure cultures of *Pseudomonas, Bacillus* and *Aeromonas* through nonspecific processes. But the bacterial isolates uptake of metals was found to be predominantly manganese when compared to other two metals chromium and nickel. *Alcaligenes* sp. was found to be more efficient in metal uptake, and it was found to tolerate all the three metals up to 500 ppm and resistant to all the antibiotics expect ampicillin. It could be interpreted from the results that within 48h through biosorption process efficient heavy metal removing capabilities and the ability to grow over a wide range of metal concentrations under aerobic conditions along with antibiotic resistance are clear indications of the advantages that may offer to employ this organism for metal remediation in simple reactors or *in situ*.

CONCLUSION

The present studies reveal that there is evidence of a relation between tolerance to heavy metals and antibiotic resistance. Thus, industrial contamination leads eventually to more resistant strains resulting in treating infections difficult causing a major global problem. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals which can be used in cleaning up or remediating metal contaminated environments.

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