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Original Article

Bacterial Tolerance to Heavy Metal Contents Present in Contaminated and Uncontaminated Soils

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Present study dealt with identification of some heavy metal tolerant bacteria from contaminated industrial soils of Dhaka Export Processing Zone (DEPZ) at Savar, tannery area at Hazaribagh and uncontaminated agricultural soils of Dhamrai and Kushtia in Bangladesh and determination of their tolerance to chromium (Cr^{6+}) and cadmium (Cd^{2+}). A total of 15 isolates from four soil samples were provisionally identified as different species of *Bacillus*, *Micrococcus* and *Pseudomonas* based on their morphological, physiological, and biochemical characteristics. Among them eight colonies were separated based on high level of heavy metal tolerance and identified at molecular level by PCR technique and 16S rRNA gene sequencing as *Micrococcus luteus* strain P43 (E4), *Bacillus pocheonensis* strain TR2-6 (T6), *Bacillus megaterium* strain H2 (T8), *Bacillus amyloliquefaciens* strain SCSAAB0007 (D10), *Bacillus cereus* isolate PGBw4 (D11), *Bacillus cereus* strain ES-4a1 (K12), *Bacillus subtilis* strain 1320, (K13), and *Bacillus subtilis* strain DP14 (K14). The Maximum Tolerable Concentration (MTC) of bacterial strains to Cr^{6+} and Cd^{2+} ranged between 250-1250 µg/ml and 30-150 µg/ml, respectively in nutrient broth medium. From the metal tolerance investigation *Bacillus* was found as the most heavy metal tolerant to both Cr^{6+} and Cd^{2+} among the three genera. The identified heavy metal tolerant bacteria could be useful for the bioremediation of heavy metal contaminated environment.

Key Words: Heavy metal tolerance, MTC, Soil bacteria, Contaminated and uncontaminated soils.

Introduction

Heavy metal pollution of soil and wastewater is a significant environmental problem[1]. In recent decades, the discharge of industrial effluents without proper treatment into soil and water bodies from different natural and anthropogenic sources containing heavy metals has resulted a serious threat to public health because of their persistence, bio-magnification, and accumulation in food chain. Most of the heavy metals like chromium, cadmium, lead, mercury and copper are highly toxic for almost all the living organisms. Each heavy metal has unique bio-functions or bio-toxicities. To investigate heavy metal tolerance of soil bacteria Cr and Cd were selected in this study based on the fact that these metals are two of important pollutants of industrial areas.

Hexavalent chromium Cr(VI) and trivalent chromium Cr(III) are the most prevalent species of chromium in the natural environment[2] and the former is more toxic for microorganisms. Major sources of Cr pollution include effluents from leather tanning, Cr electroplating, wood preservation, alloy preparation, and nuclear wastes due to its use as a corrosion inhibitor in nuclear power plants[3]. Cadmium is one of the most toxic pollutants of the surface soil layer, released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels[4].

In the contaminated sites, bacteria are continuously exposed to different heavy metals, thus giving rise to survival of metal tolerant strains. To survive under metal-stressed conditions, microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals[5]. Microbes play massive role in the biogeochemical cycling of toxic heavy metals and also in cleaning up or remediating metal-contaminated environments. Since soil is one of the most important environments for microbes and is easily exposed to many pollutants, evaluating the effects of pollutants on the microbial population is much valuable. There is increasing evidence for the evolution of metal tolerance in natural populations inhabiting contaminated sites[6]. Toxic metal tolerance in bacteria have been studied for many years but considering the range of toxic metal ions and diversity of microbes, the overall efforts appear to be limited. Therefore this study was performed to determine the heavy metal tolerance of bacteria which were isolated from metal contaminated and uncontaminated soils.

Materials and Methods

Collection of soil samples

A total of four soil samples were collected, two from contaminated sites of DEPZ, Savar and tannery area, Hazaribagh, and two from uncontaminated agricultural sites of Dhamrai and Kushtia from the surface (0-15 cm depth) in plastic bags aseptically.

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Isolation of bacteria

For the isolation of bacteria serial dilution plate technique was carried out[7]. Subsequent dilutions were made up to 10^5 times and each sample was spread over the surface of nutrient agar plates in duplicate. Plates were incubated at 37ºC for 72 hours in an incubator and colonies differing in morphological characteristics were selected and used for further studies. Three consecutive streaking of each culture were done to ensure purity of the strains.

Preparation of metal solution

Analytical grades of metal salts of $K_2Cr_2O_7$ and $CdCl_2$. $2.5H_2O$ were used to prepare 5000 µg/ml stock solutions of Cr and Cd, respectively. Each stock solution was ûlter-sterilized and added to nutrient broth at varying concentrations of metals to determine the MTC[8].

Preliminary screening of Chromium and Cadmium tolerant bacteria

The screening of previously isolated soil bacterial populations tolerant to Cr^{6+} and Cd^{2+} ions was carried out by streaking of the culture on nutrient agar plates supplemented with different concentrations of two metal ions as 0, 100, 300, 500, 1000, 1200, and 1500 µg/ml of Cr^{6+}, and 0, 30, 50, 75, 100, 150, 200, and 250 µg/ml of Cd^{2+}, separately. All plates in duplicates were incubated at 37ºC for 3 days to confirm their abilities to grow at high concentrations of Cr^{6+} and Cd^{2+} containing media. After the incubation period the concentration at which bacterial growth was present indicated bacterial tolerance to that concentration. The isolated and distinct colonies on the medium were sub-cultured repeatedly using same medium for purification.

Morphological, physiological, and biochemical characterization of the isolates

The characters of the organisms were studied using standard microbiological methods followed by Sneath *et al*.[9], SAB[10], Cappuccino and Sherman[11], Collins and Lyne[12], and Claus[13]. Colony characters such as the color, form, optical feature, margin, elevation and the shape and arrangements of vegetative cells after Gram staining[14] were observed under a phase contrast microscope from 20h old culture grown on solidified agar plates. The physiological and biochemical characters *viz*. motility, gelatin liquefaction, starch hydrolysis, indole production, oxygen requirement, oxidase test, catalase test, MR-VP test, deamination of phenylalanine, acid and gas production from D-glucose, and citrate utilization were studied.

Identification of the isolates

The isolates were tested and characterized by several microbiological key conventional tests for basic differentiation of Gram-negative and Gram-positive bacteria as described in Bergey's Manual of Determinative Bacteriology[15]. Further, the isolates were identified on the basis of biochemical tests of commercial identification systems according to Bergey's Manual of Systematic Bacteriology (Vol. 2)[9].

Identification of the isolates by PCR and 16S rRNA gene sequencing

In order to identify the isolates based on sequence comparison, partial amplification of 16S rRNA gene was done using the primer pairs of 5'-16S rRNA: CCAGACTCC TACGGGAGGCAGC and 3'-16S rRNA: CTTGTGCGGG CCCCCGTCAATTC. Polymerase Chain Reaction (PCR) products purified through alcohol precipitation were sequenced directly using a DNA auto sequencer (Applied Bio-system 3130) using the bacterial universal primers 27f and 1492r. To prepare PCR cocktail (total 400 µl for 8 samples) sterile deionized distilled water (304 µl), taq buffer B 10X (40 µl), $MgCl_2$ (24 µl), primer forward (4 µl), primer reverse (4 µl), dNTPs 10mM (4 µl), taq DNA polymerase 5U/µl (4 µl), and template DNA 25 ng/µl (16 µl) were used[16]. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI BLAST (http://blast.ncbi.nlm.nih.gov/) program to find out possible similar organism through alignment of homologous sequences in the NCBI databank.

Estimation of bacterial tolerance to metals by MTCs

For evaluation of the MTC of Cr^{6+} and Cd^{2+} of 8 bacterial isolates, nutrient broth medium in screw cap tubes with different concentrations of each metal (Cr^{6+} ranging from 200 to 1300 µg/ml and Cd^{2+} ranging from 15 to 200 µg/ml) were prepared and the bacteria were cultured readily in that tubes and kept in an incubator for 48 hours. Each liquid sample of isolates was spreaded over agar plates in duplicate and inoculated at 37ºC for 24-72h to test the appearance of growth. The presence of growth of bacterial culture was determined visually as positive or negative. The absence of bacterial growth indicated its sensitivity, while the presence of growth at certain metal concentration indicated that bacteria were tolerant to that concentration.

Results

Isolation and screening of metal tolerant bacteria

The fifteen bacterial isolates differentiated based on differences in colonial morphology were designated as mentioned in Table 1 and eight of them (E4, T6, T8, D10, D11, K12, K13, and K14) were selected as Chromium (Cr^{6+}) and Cadmium (Cd^{2+}) tolerant bacteria.

Colonial morphology

The colonial forms of all bacteria were circular; elevations were convex, effuse, and umbonate; colonial margins were regular, erose, entire, and undulate type; colonial surfaces were smooth, concentric, and rough; the colors of the colonies were yellow, brown, off-white, white, orange, and light pink. Detailed results for colonial morphology are presented in Table 2.

Table 1: *Chromium (Cr^{6+}) and Cadmium (Cd^{2+}) tolerant bacterial samples isolated from contaminated and uncontaminated soils*

Location of Soil Sample	Bacterial sample
DEPZ, Savar	E1
	E2
	E3
	E4
Tannery Area, Hazaribagh	T5
	T6
	T7
	T8
Agricultural fields of Dhamrai	D9
	D10
	D11
Agricultural fields of Kushtia	K12
	K13
	K14
	K15

Cell morphology

Cell shape of the strains were cocci, rod, and short rod whereas cell arrangements were single, paired, tetrad, chain, and scattered; all of the isolated strains were Gram positive except two; 7 isolates were non-motile and 8 were motile. Detail results for cell morphology and Gram reaction are presented in Table 2.

Biochemical characterization of the isolates

Results of physiological and biochemical tests are presented in Table 3. Seven of the isolates were facultative anaerobes and 5 were strictly aerobes. All bacterial samples showed positive results for both gelatin liquefaction and catalase tests and negative results for both indole formation and deamination of phenylalanine. Six samples showed positive and nine showed negative results for starch hydrolysis whereas nine showed positive and 6 showed negative results for oxidase tests. Among the 15 samples 8 showed positive and 7 showed negative results for MR test. Eleven isolates gave negative results and 4 were positive for VP test whereas 11 were positive and 4 were negative for the test of acid production from D-Glucose. All samples showed negative results in gas production from D-Glucose.

Conventionally Identified bacteria

Considering all observed characters of the isolated organisms, identification of Gram positive bacteria was done following Bergey's Manual of Systematic Bacteriology (Vol. 2)[9] and the results are presented in Table 4. The isolated organisms showed some minor differences in biochemical characters from those cited in that text.

Table 2. *Morphological characteristics and Gram reaction of bacterial isolates*

Morphological characteristics	Bacterial isolates														
	E1	E2	E3	E4	T5	T6	T7	T8	D9	D10	D11	K12	K13	K14	K15
Form	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Convex	Effuse	Effuse	Convex	Umbo - nate	Effuse	Convex	Effuse	Convex	Convex	Effuse	Effuse	Convex	Convex	Effuse
Margin	Erose	Undu - late	Entire	Regular	Undu - late	Entire	Entire	Entire	Entire	Undulate	Undulate	Undulate	Entire	Entire	Erose
Surface	Concentr ic	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Rough	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Color	White	White	Off White	Yellow	White	Brown	Brown	Off White	Off White	Off White	Off White	White	Light Pink	Light Pink	Orange
Shape and arrange - ment of cells	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Cocci, rounded end, occur in tetrads	Rod, rounded end, occur in chain	Rod, rounded end, occur in single	Short Rod, rounded end, occur in single	Rod, rounded end, occur in chain	Short Rod, rounded end, occur in single	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Short Rod, rounded end, occur in single
Motility	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+

Table 3. *Biochemical characteristics of bacterial isolates*

Biochemical Characteristics	Bacterial Samples														
	E1	E2	E3	E4	T5	T6	T7	T8	D9	D10	D11	K12	K13	K14	K15
Oxidase test	+	-	-	+	-	+	+	-	+	+	-	-	+	+	+
Catalase Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxygen Requirement	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Strictly Aerobes	Strictly Aerobes	Facultative Anaerobes	Strictly Aerobes	Facultative Anaerobes	Strictly Aerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Strictly Aerobes
Gelatin Liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-
Indole Formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP Test	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-
MR Test	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
Deamination of Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of Citrate	-	+	+	+	+	-	-	+	+	-	+	+	+	+	-
Acid Production from D-Glucose	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+
Gas Production from D-Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. *Name of the identified bacteria*

Isolates	Name of the identified isolates
E1	*Bacillus lentus*
E2	*Bacillus cereus*
E3	*Bacillus pumilus*
E4	*Micrococcus luteus*
T5	*Bacillus firmus*
T6	*Bacillus pocheonensis*
T7	*Pseudomonas pseudoalcaligenes*
T8	*Bacillus megaterium*
D9	*Pseudomonas pseudoalcaligenes*
D10	*Bacillus amyloliquefaciens*
D11	*Bacillus cereus*
K12	*Bacillus cereus*
K13	*Bacillus subtilis*
K14	*Bacillus subtilis*
K15	*Bacillus globisporus*

Identification of bacteria based on PCR and 16S rRNA gene sequencing analysis

Eight previously selected metal tolerant bacterial isolates were used for further confirmation test by PCR and 16S rRNA gene sequencing. The most closely related sequences were found using the BLAST programs against similar sequences in the NCBI databank and isolate E4 was affiliated to *Micrococcus luteus* strain P4_3 (99% similarity), isolate T6 to *Bacillus pocheonensis* strain TR2-6 (99% similarity), isolate T8 to *Bacillus megaterium* strain H2 (99% similarity), isolate D10 to *Bacillus amyloliquefaciens* strain SCSAAB0007 (99% similarity), isolates D11 to *Bacillus cereus* isolate PGBw4 (99% similarity), isolate K12 to *Bacillus cereus* strain ES-4a1 (99% similarity), isolate K13 to *Bacillus subtilis* strain 1320 (99% similarity), isolate K14 to *Bacillus subtilis* strain DP14 (99% similarity).

Determination of MTC against Chromium and Cadmium

The eight strains selected as heavy metal tolerant bacteria were used for evaluation of MTC. The MTC of all the bacterial strains against Cr^{6+} and Cd^{2+} have shown that the strains were capable of growing at high concentrations of heavy metal ions in nutrient broth and the results of the experiments are given in Table 5. The microbial load decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms.

Table 5. *Maximum Tolerable Concentrations (MTC) against Chromium (Cr^{6+}) and Cadmium (Cd^{2+}) of each bacterium cultured in nutrient broth*

Isolates	Identified Bacterial strain	MTC against Cr^{6+} (µg/ml) in Nutrient broth	MTC against Cd^{2+} (µg/ml) in Nutrient broth
E4	*Micrococcus luteus* strain P4_3	600	50
T6	*Bacillus pocheonrncis* strain TR2-6	250	120
T8	*Bacillus megaterium* strain H2	800	150
D10	*Bacillus amyloliquefaciens* strain SCSAAB0007	1000	30
D11	*Bacillus cereus* isolate PGBw4	1250	30
K12	*Bacillus cereus* strain ES-4a1	1250	75
K13	*Bacillus subtilis* strain 1320	950	75
K14	*Bacillus subtilis* strain DP14	1000	50

Discussion

Sampling sites were selected with the aim to isolate metal tolerant bacteria for which the best option was to locate metal contaminated sites. Uncontaminated sites were also selected to evaluate the tolerable limits of heavy metals of common soil bacteria. Bacteria exposed to high levels of heavy metals in their environment have adapted to this stress by developing various resistance mechanisms which could be utilized for detoxification and removal of heavy metals from polluted environment[17]. However, 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 non-polluted sites and not in all cases is any adaptation necessary[18]. Such resistance mechanisms are the basis for the use of microorganisms in bioremediation approaches.

The current study revealed that heavy metal tolerant bacteria were isolated from both contaminated and uncontaminated soils and eight Gram positive bacteria showed the same pattern of metal tolerance against Cr^{6+} and Cd^{2+} in the order of $Cr^{6+} > Cd^{2+}$ according to the values of MTC. Hence, Cr^{6+} was found to be more tolerable metal ion whereas Cd^{2+} appeared to be highly toxic to the bacterial strains. Among eight bacteria maximum tolerance to Cr^{6+} was shown by *Bacillus cereus* isolate PGBw4 (D11) isolated from agricultural field of Dhamrai and *Bacillus cereus* strain ES-4a1 (K12) isolated from agricultural soil of Kushtia showing growth up to 1250 µg/ml and maximum tolerance to Cd^{2+} was shown by *Bacillus megaterium* strain H2 (T8) isolated from tannery area showing growth up to 150 µg/ml. Besides, minimum tolerance to Cr^{6+} was shown by *Bacillus pocheonensis* strain TR2-6 (T6) isolated from tannery area showing growth up to 250 µg/ml and minimum tolerance to Cd^{2+} was shown by *Bacillus amyloliquefaciens* strain SCSAAB0007 (D10) and *Bacillus cereus* isolate PGBw4 (D11) isolated from agricultural field of Dhamrai showing growth up to 30 µg/ml.

The results showed that different bacterial strain of same species could show different MTC to heavy metals. It might be due to variation in genetic level since metal resistance mechanisms of bacteria are sometimes gene-regulated. The order of tolerance to Cr^{6+} of identified all Gram positive bacterial strain of *Micrococcus* and *Bacillus* spp. is *B. cereus* > *B. amyloliquefaciens* > *B. subtilis* > *B. megaterium* > *Micrococcus luteus* > *B. pocheonensis* and the order of tolerance to Cd^{2+} is *B. megaterium* > *B. pocheonensis* > *B. subtilis* > *Micrococcus luteus* > *B. amyloliquefaciens* > *B. cereus.* In this study it was found that all Gram positive bacteria showed higher Cr^{6+} tolerance. It was reported that Gram-positive bacteria were more Cr^{6+} tolerant than Gram-negative bacteria[19]. This was also reported by other authors who widely documented the high presence of tolerant Gram-positive bacteria in soil polluted with heavy metals[20]. Other results have also reported that the diversity of *Bacillus* was greatest in contaminated soil[21].

Conclusion

Among the genera of *Bacillus*, *Pseudomonas*, and *Micrococcus* identified in the present study *Bacillus* was found as the most heavy metal tolerant bacteria having high degree of tolerance against Chromium and Cadmium both in contaminated and uncontaminated soils whereas others were relatively susceptible. It indicates their easy survival capacity in metal polluted environment. Hence *Bacillus* will be common in microbial composition of uncontaminated agricultural soils and will regulate the transformation mechanisms of those soils though soils are being contaminated with heavy metals as because soil transformation processes are largely dependent on relevant microbial population. The abundance of *Bacillus* was possibly due to the spore structure in the *Bacilli* group which increases its power of uptake and resistance against the metals. Evaluation of heavy metal tolerance in bacteria may provide a great insight into application of processes in bioremediation. Bacterial tolerance to heavy metals may be a fall out of the detoxification mechanisms intrinsic to the bacteria. Future studies should investigate heavy metal tolerance in bacteria in industrially contaminated soil ecosystems and may demonstrate its utility in detecting environmental pollution by heavy metals, transformation of nutrients in soils, and for element recovery and environmental protection by economically feasible and technologically efficient means.

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