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Isolation and characterization of heavy metal tolerant Gram-positive bacteria with bioremedial properties from municipal waste rich soil of Kestopur canal (Kolkata), West Bengal, India

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Abstract: The present study was conducted to determine the culturable bacterial profile from Kestopur canal (Kolkata, India) and analyze their heavy metal tolerance. In addition to daily sewage including solid and soluble wastes, a considerable load of toxic metals are released into this water body from industries, tanneries and agriculture, household as well as health sectors. Screening out microbes from such an environment was done keeping in mind their multifunctional application especially for bioremediation. Heavy metals are major environmental pollutants when present in high concentration in soil and show potential toxic effects on growth and development in plants and animals. Some edible herbs growing in the canal vicinity, and consumed by people, were found to harbour these heavy metals at sub-toxic levels. The bioconcentration factor of these plants being <1 indicates that they probably only absorb but not accumulate heavy metals. All the thirteen Gram-positive bacteria isolated from these plants rhizosphere were found to tolerate high concentration of heavy metals like Co, Ni, Pb, Cr, Fe. Phylogenetic analysis of their 16S rDNA genes revealed that they belonged to one main taxonomic group – the Firmicutes. Seven of them were found to be novel with 92–95% sequence homology with known bacterial strains. Further microbiological analyses show that the alkaliphilic *Bacillus weihenstephanensis* strain IA1 and *Exiguobacterium aestuarii* strain CE1, with selective antibiotic sensitivity along with high Ni^{2+} and Cr^{6+} removal capabilities, respectively, can be prospective candidates for bioremediation.

Key words: antibiotic resistance; biochemical assay; growth curve; heavy metal tolerance; pH tolerance; 16S rDNA.

Abbreviations: BCF, bioconcentration factor; LB, Luria Bertani; MIC, minimum inhibitory concentration.

Introduction

Heavy metals such as lead (Pb), chromium (Cr), cadmium (Cd), mercury (Hg), iron (Fe), cobalt (Co) and nickel (Ni) are important environmental pollutants, particularly in areas with high anthropogenic pressure. Their presence in atmosphere, soil and water, even in traces, can cause serious problem to all organisms (Islam et al. 2007). Heavy metal contamination of the food items is one of the most important aspects of food quality assurance (Wang et al. 2005; Khan et al. 2008). International and national regulations on food quality have lowered the maximum permissible levels of toxic metals in food items due to an increased awareness of the risk, these metals pose to food chain contamination. Rapid and unorganized industrialization and urbanization have contributed to the elevated level of heavy metals in the urban environment of the developing countries, such as China (Wong et al. 2003) and India (Khillare et al. 2004; Sharma et al. 2008).

Heavy metals and their derivatives are non-biodegradable and persistent environmental contaminants which may be deposited on the surfaces and then adsorbed into the tissues of the vegetables. Vegetables constitute an important part of the human diet since they contain carbohydrates, proteins, vitamins, minerals and fibres required for human health. They also act as neutralizing agents for acidic substances formed during digestion. Plants take up heavy metals by absorbing them from deposits on the parts of the plants exposed to the air from polluted environment as well as from contaminated soils (Khairiah et al. 2004; Al-Jassir et al. 2005; Sharma et al. 2008). In many developing countries it is a common practice to grow vegetables along banks of rivers passing through urban centres. Waters of such rivers have often been reported to be polluted by heavy metals (Mashauri & Mayo 1990; Kashem & Singh 1999; Othman 2001). The extent of absorption of the elements by the plant depends on, among other things, the nature of the plant, chemical constitution of the pol-

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lutant, concentration of the element in the soil, pH and the interaction with other metals (Zurera-Cosano et al. 1989). The risk of contaminants accumulating in soil, environment and crops due to sewage water, fertilizer and pollutants is of serious concern. Heavy metals have been reported to produce mutagenic, teratogenic, neurotoxic and carcinogenic effects even at very low concentrations (Waalkes et al. 1994; Al-Saleh et al. 1996). Human beings have also been known to develop several diseases like cardiovascular problems, tubular dysfunction in kidneys and nervous disorders due to metal toxicity.

Exploring the microbial habitats having elevated level of heavy metals has 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. The metal accumulating capacity of microorganisms can be exploited to remove, concentrate and recover metals from mine tailings and industrial effluents (Malekzadeh et al. 2002). Several reports of aerobic bacteria accumulating metals like Ag, Co, Cd, Cu, Cr, and Ni (Mengoni 2001; Chovanová et al. 2004; Adarsh et al. 2007; Karelová et al. 2011) are available. Microorganism-based remediation techniques, such as bioremediation, show potential for their ability to degrade and detoxify certain contaminants (Sherameti & Varma 2011).

This study reports the isolation and characterization of some bacteria, isolated from the soil rhizosphere, collected from the banks of Kestopur canal, which runs through the northern fringes of Kolkata, India. This canal receives effluents from domestic activities, industries, tanneries, battery manufacturing units as well as health sectors. The hot and humid climate all throughout the year favours this site to act as an incubator for diverse group of microbes. Owing to steep growth in population and urbanization along its stretch, the water flow in the area is deeply impeded. Most of the people inhabiting this area are poor and hence use this as source of water for household activities. They also cultivate vegetables like *Colocasia esculenta*, *Coccinia cordifolia*, *Amaranthus viridis*, *Cleome rutidospermum*, *Ipomea aquatica* and *Alternanthera sessilis* along the soil surrounding the canal, which form the main source of food for these people. Thus the site was selected to study the wide variety of metal-tolerant microbes from the rhizosphere of these plants and explore their potentialities as bioremediators. Out of the thirteen isolated heavy metal tolerant strains, *Exiguobacterium profundum* strain CC1 (as isolated from rhizosphere of *Coccinia cordifolia*), *Bacillus anthracis* strain AS1 and *Bacillus mycoides* strain AS2 (as isolated from rhizosphere of*Alternanthera sessilis*) showed very high degree of antibiotic resistance. Further characterization of these strains, in terms of pathogenic potential, are very important as they may pose serious threat to the human population. Our results also indicate that alkaliphilic *Bacillus weihenstephanensis* strain IA1 (as isolated from rhizosphere of *Ipomea aquatica*) and *Exiguobacterium aestuarii* strain CE1 (as isolated from rhizosphere of *Colocasia esculenta*) with selective antibiotic sensitivity along with high Ni^{2+} and Cr^{6+} removal capabilities, respectively, can be prospective candidates for bioremediation.

Material and methods

Field site, soil samples and plant materials

Six vegetables (*Colocasia esculenta*, *Coccinia cordifolia*, *Amaranthus viridis*, *Cleome rutidospermum*, *Ipomea aquatica* and *Alternanthera sessilis*) and samples of rhizosphere soil down to 10 cm depth, were collected randomly in triplicate (18 samples) from the banks of Kestopur canal, which runs through the northern fringes of Kolkata, India (coordinates: 22°35′31″ N and 88°25′51″ E).

Determination of heavy metal concentration in soil

Ten g each of soil samples were collected in a triangular array from three points in the rhizosphere of *Colocasia esculenta*, *Coccinia cordifolia*, *Amaranthus viridis*, *Cleome rutidospermum*, *Ipomea aquatica* and *Alternanthera sessilis*. All were stored in black polythene bags before digestion and analysis. The soil samples were fine to medium sand in texture. They were ground, mixed and divided into fine particles that could pass through a 0.5 mm sieve. Digestion was done by placing 2 g of soil in a conical flask, adding 15 mL of concentrated nitric acid and perchloric acid at a ratio 1:1 and heating in a fume cupboard at a temperature of 105 °C until the volume was reduced to slightly less than 1 mL, then the sample was allowed to cool, 10 mL of deionised water were added, and the solution stirred and filtered and made up in standard volumetric flask. The results were the average of three measurements per plant. Metal contents of samples were measured in an air-acetylene flame by atomic absorption spectrophotometry using a double-beam optical system with deuterium arc background correction (AAnalyst 100; Perkin-Elmer, UK).

Determination of heavy metal concentration in plants

For determination of whole-leaf metal concentrations, fresh plant material was oven-dried at 80 °C for 48 h. Subsamples of 50 mg of dried leaf material were digested in 3 mL of concentrated (69%, v/v) nitric acid for 16 h in glass vials. Samples were then diluted 10-fold with ultrapure water and filtered using Whatman grade 3 filter paper. Metal contents of samples were measured in an air-acetylene flame by atomic absorption spectrophotometry using a double-beam optical system with deuterium arc background correction (AAnalyst 100; Perkin-Elmer, UK). Samples were further diluted as necessary to fall within the linear range of calibration curves prepared using appropriate standard solutions and reagent blanks. Three technical replicates were analysed for each measurement, and two samples from each of three plants were analysed for each metal treatment. Metal accumulation and uptake by living organisms in the soil are also given by the 'bioconcentration factor' (BCF). It provides an index of the ability of the organisms to accumulate a particular metal with respect to its concentration in the substrate (Zayed et al. 1998). It was calculated as follows: $BCF = HM_{plant}/HM_{soil}$ [HM = Heavy metal content in plant part or soil].

Isolation of bacterial strains
A 10 g portion (wet weight) of the soil was mixed in a sterile 250 mL Erlenmeyer flask with 90 mL of a 0.85% (w/v) salt solution and incubated at 30 °C in a shaker incubator at 90 rpm for 2 h. The suspensions obtained were filtered through Whatman 1 filter paper (Merck, Germany) under sterile conditions, and these filtered bacterial suspensions were used for further work. Subsamples (1.0 mL) withdrawn from the soil-borne bacterial suspension were serially diluted (in range: 10^{-1}–10^{-6}) and each dilution was plated in triplicate on Luria Bertani (LB) agar plate. The plates were incubated aerobically at 37 °C for either 24–48 h. Individual pure colonies were screened on LB agar media containing tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L in double distilled water with different heavy metal salts, like $Pb(NO_3)_2$, $NiSO_4 \cdot 6H_2O$, $HgCl_2$, $K_2Cr_2O_7$, $CoCl_2 \cdot 6H_2O$, $CdCl_3$, and $FeSO_4 \cdot 7H_2O$. From the independently growing colonies, thirteen different strains were randomly selected (on the basis of metal tolerance) for further analysis. Further cultivation and maintenance of pure isolates was done in LB broth. Liquid cultures were incubated at 37 °C with constant shaking at 120 rpm. Isolated strains were stored at −80 °C in culture medium containing 70% (v/v) glycerol.

Temperature dependent bacterial growth measurement
Since pH and temperature are limiting factors governing the microbial growth, determination of the optimum conditions for these parameters are necessary. For temperature profile, the culture (1%) was grown in LB broth and was incubated for 12 h at 20, 30, 37 and 40 °C at 150 rpm shaking condition. The optical density measured in UV-VIS spectrophotometer (Shimadzu UV-VIS spectrophotometer) at 595 nm gave the growth efficiency. To find the pH range, LB broths of different pH were prepared using NaOH and HCl for acidic and alkaline range, respectively. A 1% inoculum was provided to medium of each pH range and the bacterial culture was incubated for 12 h at the optimum temperature found for each isolate. Selected strains were optimized at five different values of pH (3, 5, 7, 9, and 11). The presence or absence of growth was defined in terms of optical density at 660 nm (UV-VIS Spectrophotometer).

Heavy metal tolerance determination
Metal salts of $Pb(NO_3)_2$, $NiSO_4 \cdot 6H_2O$, $HgCl_2$, $K_2Cr_2O_7$, $CoCl_2 \cdot 6H_2O$, $CdCl_3$, and $FeSO_4 \cdot 7H_2O$ were used for tolerance study. Stock solution of each metal was prepared on basis of molar concentration as per formulae 'M = Moles of solute/Volume of solution'. From an overnight grown culture of a single colony 1% (v/v) was transferred to 3 mL of media supplemented with each metal separately. The initial metal concentration was taken as 1 mM. The tolerance was measured on the basis of growth observed (turbidimetry method) within 12–48 h. If growth was observed, inoculum was added to the media with increasing concentration of the metal. This step was repeated for all the metals in each strain till minimum inhibitory concentration (MIC) was obtained as visualized by cessation of growth (Yilmaz 2003).

Optimization of pH and temperature for heavy metal removal from culture media
Exiguobacterium strain CE1 and *Bacillus* strain IA1 were inoculated into 250 mL conical flasks at different pH containing either 100 mg/L of chromium or 50 mg/L of nickel, respectively. After 24 h incubation, heavy metal (Cr and Ni) removal and biomass were measured according to Congeevaram et al. (2007).

Morphological characterization of bacterial strains
Different cultural parameters like growth form and elevation were observed in LB agar plates after incubation at 37 °C for 2–3 days. Growth form, edges, colour, etc., of the bacterial colonies were observed within plate. The cellular morphology of a Gram stained preparation was determined at 100× magnification by bright field microscope on a Zeiss Axiostar Plus microscope.

Biochemical characterization of bacterial strains
Presence of certain extracellular enzymes like amylase, DNase, catalase and pectinase were confirmed following the biochemical characterization techniques as reported by Nandy et al. (2007). Specific ready-made media (HiMedia Laboratory) were used for detection of enzymes, like DNase. Pectinase was detected on minimal media supplemented with 1% pectin where the enzyme, if present, produces a clean zone around the colony. Catalase tests are based on the basic property of the enzyme and were done on LB plates containing single colony of pure isolates.

Antibiotic sensitivity test
The Mueller-Hinton agar medium (Media No. M147 HiMedia) was prepared and commercially available antibiotic disks from HiMedia were used. The strains were grown upto mid log phase, diluted upto 10^{-2} times; spread on Mueller-Hinton agar plates and antibiotic discs were placed. The plates were incubated overnight at 37 °C and observed for zone of inhibition. The 10 different antibiotic disks used were cefotaxime (30 μg), ampicillin (10 μg), norfloxacin (10 μg), trimethoprim (30 μg), doxycycline (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), rifampicin (15 μg), and metronidazole (4 μg). The sensitivity and resistance profile was determined based on the diameter of the zone of inhibition and evaluation done according to National Committee for Clinical Laboratory Standard's (NCCLS) chart provided with the antibiotic kits by HiMedia.

Bacterial growth profile measurement
From an overnight confluent culture of a single colony, 1% inoculum was given in LB broth and the growth was monitored at regular intervals by measuring the optical density at 595 nm in a spectrophotometer (Shimadzu UVPC).

Genomic DNA isolation
Genomic DNA was isolated with microbial DNA isolation kit (MOBIO) according to manufacturer's protocol.

PCR amplification of 16S rDNA
DNA extracted from soil bacterial isolates was used in PCR either with universal 16S rDNA gene primer set (27F and 1492R). Each 50 μL reaction mixture contained 2 μL of the DNA template, 5 μL 10X Taq buffer (Qiagen, Hilden, Germany), 2.5 U Taq DNA polymerase (Hot-Start; Qiagen, Germany), 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates and 0.5 μM of each primer. PCRs were performed in a thermal cycler (Eppendorf) with the following cycling conditions: 4 min of denaturation at 95 °C, 25 cycles of 1 min at 94 °C, 1 min at 53 °C, 1.5 min at 72 °C, and a final cycle of extension at 72 °C for 5 min. PCR products were analyzed by electrophoresis in a 1% (w/v) agarose gel and purified using the MOBIO Gel Extraction Kit according to the manufacturer's instructions. PCR products were sequenced with Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) in accordance with the manufacturer's protocol and then sequenced using ABI 16S rDNA sequencing Kit.

Table 1. Concentration of heavy metals in the soil.[a]

Heavy metal	Cr	Pb	Co	Ni	Fe
Soil	67.87±0.21	72.5±4.11	17.94±0.19	25.67±0.17	37.53±0.63
Standard values	100	250–500	50	75–150	–

[a] Concentrations are expressed in mg/kg. Extraction of ions and its measurement in variance atomic absorption spectrophotometer is described in Material and Methods. The results obtained from three separate experiments were calculated from the standard solutions of metal ions (±S.E.). The standard values are according to Awashthi (2000).

Table 2. Concentration of heavy metals in the edible plants.[a]

Heavy metal	Cr	Pb	Co	Ni	Fe
λ(nm)	357.9	217	240.7	232	248.3
Cleome rutidospermum	43.87±0.01	59.5±0.83	11.32±0.14	17.67±0.59	23.94±0.04
Ipomea aquatic	39.42±0.39	61.55±0.76	17.26±0.53	16.02±0.11	29.27±0.54
Colocasia esculenta	46.29±0.54	48.46±0.36	09.92±0.18	23.43±0.07	27.62±0.58
Coccinia cordifolia	45.25±0.72	46.72±0.63	16.46±0.50	21.31±0.79	21.68±0.53
Amaranthus viridis	42.07±0.44	60.05±0.46	07.45±0.17	20.83±0.43	33.74±0.64
Alternanthera sessilis	41.93±0.63	53.78±0.92	10.80±0.39	16.09±0.78	28.66±0.52
Standard value	30	30–300	5	2	–
Bioconcentration factor					
Cleome rutidospermum	0.64	0.82	0.63	0.69	0.63
Ipomea aquatic	0.58	0.80	0.96	0.62	0.78
Colocasia esculenta	0.65	0.63	0.55	0.91	0.73
Coccinia cordifolia	0.63	0.60	0.91	0.83	0.57
Amaranthus viridis	0.58	0.78	0.41	0.81	0.90
Alternanthera sessilis	0.58	0.70	0.60	0.62	0.76

[a] Concentrations are expressed in mg/kg. Extraction of ions and its measurement in variance atomic absorption spectrophotometer is described in Material and methods. The results obtained from three separate experiments were calculated from the standard solutions of metal ions (±S.E.). The standard wavelength (λ) was used for determination of different heavy metal (Center for Environmental Research, Information Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268, 1999). The standard values for different heavy metals are from Kabata-Pendias & Pendias (1992). The bioconcentration factor was calculated as the ratio of content of heavy metal in plants to that in the soil.

Bacterial strain identification and phylogenetic analysis

Specific 16S rDNA sequences were edited with Chromas Lite software (version 2.01) for further DNA analysis. A BLAST search (Altschul et al. 1990; Zhang et al. 2000) at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to identify the nearest neighbours. Multiple alignments were performed with the ClustalW2 program (Larkin et al. 2007) at the EBI server (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic tree was generated with the MEGA5 software (Tamura et al. 2011) on the basis of evolutionary distances calculated by the neighbour-joining method (Saitou & Nei 1987) using Maximum Composite Likelihood model (Tamura et al. 2004).

Nucleotide sequence accession numbers

The 16S rDNA partial gene sequences generated in this study have been deposited with the GenBank database (Benson et al. 2012) under the accession numbers JN392001–JN392013.

Statistical analysis

All experiments were performed in triplicate. The statistical calculation was done according to the standard method. The results are given as mean ± SD values.

Result and discussion

Characterization of soil and water

The pH value of the soil from where bacterial strains were collected was 8.03. The colour of the soil was grayish black and texture more or less smooth and pH of the canal water was 8.09.

Analysis of heavy metal concentration in soil and edible herbs

High concentrations of heavy metals (Table 1) especially lead and chromium in the soil, which is above the threshold value, is of potential threat to human population living in this area. The amount of metal accumulated in the edible plants was studied to find out their toxicity level. Results (Table 2) clearly show that the six plants studied had different level of heavy metal accumulation. While *Ipomea* and *Coccinia* showed highest amount of Co accumulation (BCF = 0.96 and 0.91, respectively), all the six genera absorbed high amount of Cr as well, ranging between 39.42–46.29 mg/kg. The amount of heavy metal accumulated based on BCF analysis shows that *Cleome rutidospermum* accumulated more Pb, *Colocasia esculenta* accumulated Ni while *Amaranthus viridis* and *Alternanthera sessilis* accumulated more Fe. However, the comparatively low level accumulation of heavy metal in plant species studied can be attributed to the association of bioremediating microbes in the rhizosphere. Huang et al. (2005) had inferred that mycorrhiza protect its host plants from the phytotoxicity of excessive copper, zinc and lead by changing the speciation from bioavailable to the

Table 3. Molecular identification of the isolates based on partial 16S rDNA analysis.[a]

Strain	Bacterial strain showing maximum homology	Identity (%)	GenBank
CC1	*Exiguobacterium profundum* strain 10C	99	JN392008
CC2	*Exiguobacterium marinum* strain TF-80	97	JN392001
AV1	*Bacillus thuringiensis* strain IAM 12077	99	JN392009
AV2	*Bacillus stratosphericus* strain 41KF2a	99	JN392003
IA1	*Bacillus weihenstephanensis* strain DSM11821	99	JN392010
IA2	*Bacillus aerophilus* strain 28K	96	JN392002
CE1	*Exiguobacterium aestuarii* strain TF-16	99	JN392011
CE2	*Exiguobacterium mexicanum* strain 8N	96	JN392004
AS1	*Bacillus anthracis* strain ATCC 14578	97	JN392012
AS2	*Bacillus mycoides* strain 273	98	JN392013
CR1	*Bacillus acidicola* strain 105-2	95	JN392005
CR2	*Bacillus marisflavi* strain TF-11	93	JN392006
CR3	*Bacillus luciferensis* strain LMG 18422	92	JN392007

[a]The sequence was subjected to BLAST analysis and the identity was deciphered on the basis of the closest neighbour within the existing database showing maximum % of identity. The sequences were submitted to GenBank with the respective accession numbers. The strains were designated according to their site of isolation from the rhizosphere of the respective plants. CC – *Coccinia cordifolia*, AV – *Amaranthus viridis*, IA – *Ipomea aquatica*, CE – *Colocasia esculenta*, AS – *Alternanthera sessilis*, CR – *Cleome rutidospermum*.

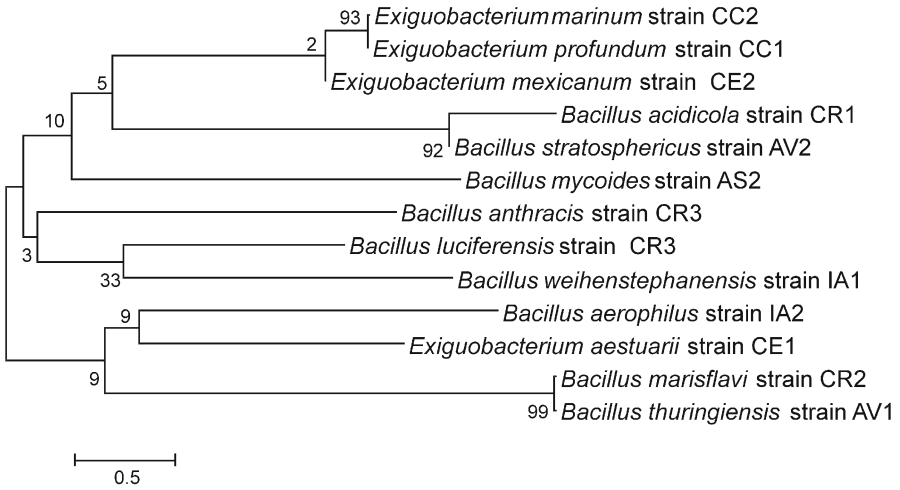

Fig. 1. Phylogenetic tree based on 16S rDNA sequences. The evolutionary distance was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 16.63905902 is shown.

non-bioavailable form. Soil pollution with heavy metals leads to the appearance of heavy-metal resistant rhizobateria in the soil of industrial regions (Aleem et al. 2003). Karelová et al. (2011) performed a phylogenetic analysis of a culturable bacterial community isolated from heavy-metal contaminated soil from southwest Slovakia using 16S rDNA and heavy-metal resistance genes. Many studies have focused on the effects of heavy metals on bacterial community structure (Khan et al. 2010; Karelová et al. 2011), and relatively many bacteria have already been isolated from different heavy-metal-contaminated environments. Therefore, the objective of this study was to isolate and characterize plant-rhizosphere specific heavy-metal tolerant bacteria and explore their potentials in metal prospecting and bioremediation.

Analysis of bacterial isolates on the basis of their 16S rDNA sequencing

The isolated heavy metal tolerant bacteria were identified based on their nearest bacterial relatives using phylogenetic analysis. The 16S rDNA sequences obtained for the isolates were subjected to megaBLAST. Strains with low similarity to their close relatives were considered to belong to new species or genera. The strains showed 92–99% sequence identity to different strains of *Exiguobacterium* and *Bacillus* (summarised in Table 3). The 16S rDNA sequences from the 13 strains were submitted to GenBank and are available under the accession numbers JN392001–JN3920013. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (Fig. 1) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd}+Noncoding. All positions containing gaps and missing data were eliminated. There were a total

Table 4. Minimum inhibitory concentration (mM) of different metals obtained for the 13 isolates from Keshtopur canal.

Strain	Cr	Pb	Co	Ni	Fe
Exiguobacterium profundum strain CC1	9	5	0.9	0.6	8
Bacillus thuringiensis strain AV1	7	8	0.6	0.7	9
Bacillus weihenstephanensis strain IA1	8	7	0.6	0.8	9
Exiguobacterium aestuarii strain CE1	9	6	0.8	0.6	7
Bacillus anthracis strain AS1	8	8	0.7	0.7	8
Bacillus mycoides strain AS2	7	7	0.6	0.6	7
Exiguobacterium marinum strain CC2	9	5	0.9	0.6	7
Bacillus aerophilus strain IA2	7	8	0.7	0.5	9
Bacillus stratosphericus strain AV2	8	8	0.7	0.7	9
Exiguobacterium mexicanum strain CE2	9	5	0.9	0.5	7
Bacillus acidicola strain CR1	8	7	0.8	0.6	8
Bacillus marisflavi strain CR2	7	7	0.7	0.6	9
Bacillus luciferensis strain CR3	7	6	0.7	0.7	9

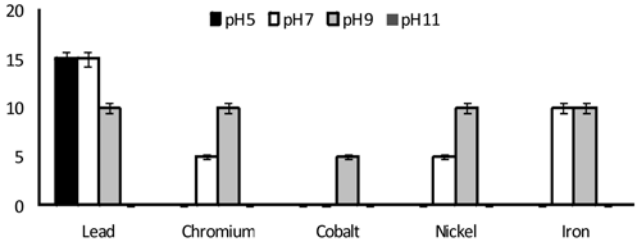

Fig. 2. Growth of bacterial strains in presence of metals at different pH. LB broth of different pH were prepared using NaOH and HCl for acidic and alkaline range respectively. The inoculum (1%) was provided to medium of each pH range and the bacterial culture was incubated for 12 h at the optimum temperature found for each isolate. Selected strains were optimized at five different pH values (3, 5, 7, 9, 11).

of 698 positions in the final dataset. Evolutionary analyses were conducted using the MEGA5 (Tamura et al. 2011).

Determination of MIC, optimal temperature and pH The preliminary characterization of the thirteen identified Gram-positive bacterial isolates was done on the basis of their tolerance towards Pb, Co, Ni, Cr and Fe (Table 4). While all the isolates showed complete inhibition at 10 mM concentration, MIC of Cr and Fe tolerant strains ranged from 7–9 mM, Pb tolerant strains had MIC ranging from 5–8 mM, while MIC for Co/Ni tolerant strains were 0.5–0.9 mM. Bacterial strain *Exiguobacterium* sp. ZM-2 isolated from agricultural soil irrigated with tannery effluents, was reported by Alam & Malik (2008) for its resistance to hexavalent chromium. The biosorption capacities of *Bacillus cereus* and *Bacillus pumilus* were observed by Colak et al. (2011). In accordance with previous studies in our survey, also *Exiguobacterium* sp. mainly showed Cr and Co tolerance, while *Bacillus* sp. exhibited high level of Pb, Ni and Fe tolerance. Interestingly, lead tolerant bacteria also showed maximum growth at pH 5 and 7, cobalt tolerant *Exiguobacterium* sp. grew only at pH 9 (Fig. 2) revealing its alkaliphillic nature (Vishnivetskaya et al. 2009). The bacterial media at pH 9 was suitable for almost all Cr and Ni resistant bacteria and was considered as standard pH for these bacterial strains.

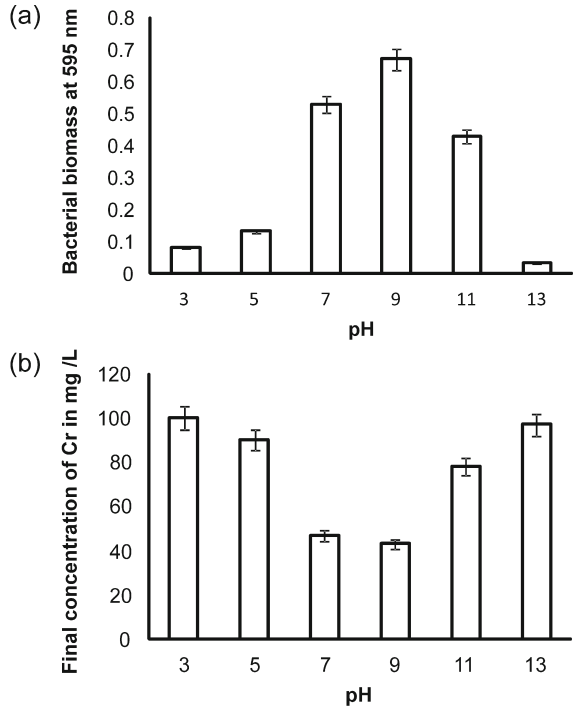

Fig. 3. (a) Cellular growth and (b) Cr (VI) removal by *Exiguobacterium* sp. in response to various pH. Temperature: 37 ℃, incubation time: 24 h, concentration of Cr (VI): 100 mg/L. Each point represents the mean of values obtained from five separate experiments ($n = 5$) ± 1% standard error.

Study of metal removing properties of Bacillus sp. and Exiguobacterium sp. in alkaline condition Having established the ability of these bacteria to tolerate heavy metals, work was carried out to establish whether they were able to remove heavy metals from culture at high pH *in vitro*. In the pH range studied (3–13 for 100 mg/L of chromium and 50 mg/L of nickel), maximum removal of Cr (VI) (50%) was observed at around pH 9 by *Exiguobacterium* sp. (Fig. 3), while *Bacillus* sp. was able to remove 70% of Ni (II) from culture media at similar pH (Fig. 4). With increase in pH from 3 to 5 almost no bioaccumulation occurred. This low bioaccumulation capacity at pH values below five is attributed to the competition of hydrogen ion with metal ion on the sorption site. Most of the microbial surfaces

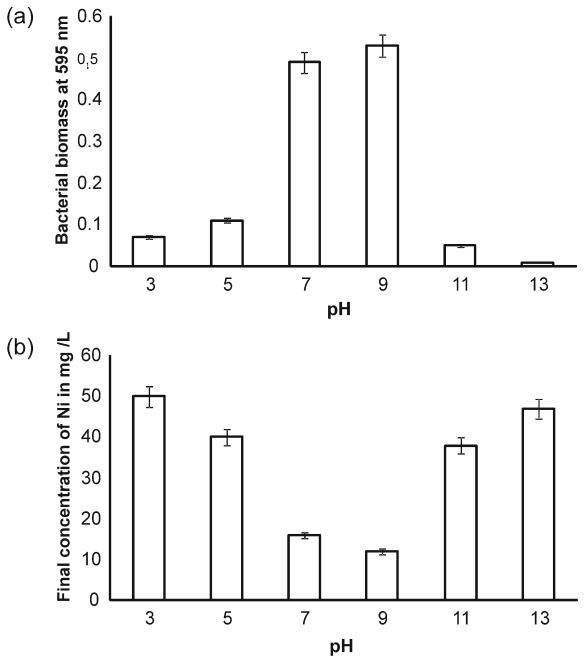

Fig. 4. (a) Cellular growth and (b) Ni (II) removal by *Bacillus* sp. in response to various pH. Temperature: 37 ℃, incubation time: 24 h, concentration of Ni (II): 50 mg/L. Each point represents the mean of values obtained from five separate experiments ($n = 5$) ± 1% standard error.

are negatively charged due to the ionization of functional groups, thereby contributing to metal binding. With increase in pH, there is increase in metal removal, due to the ionization of functional groups and an increase in the negative charge density on the cell surface (Congeevaram et al. 2007). Hasan et al. (2000) reported maximum removal of nickel in the pH range of 4.5–5.5. Yan & Viraraghavan (2003) reported similar results by *Mucor rouxii* for the removal of lead. Low removal of nickel at lower pH range by *Pencillium chrysogenum* has also been reported (Tian-Wei et al. 2004). Removal of copper and lead (Leung et al. 2000) and chromium and nickel (Congeevaram et al. 2007) by *Micrococcus luteus* also followed the same trend. Very few bacterial species are known to remove metals at pH above 8. As the pH is increased above the zeta potential of the adsorbent, reduction in the electrostatic attraction between the Cr (VI) species and the sorbent surface, with a consequent decrease in percentage bioaccumulation occurs (Congeevaram et al. 2007). In our study *Exiguobacterium* sp and *Bacillus* sp showed maximum growth and removal of chromium and nickel at higher alkaline pH values (8 and above) (Figs 3 and 4). The positive correlation between heavy metal removal and cellular growth, consistently observed regardless of the kind of heavy metal and pH, strongly suggests that the observed effect is based not only on the metal chemistry in solution or the surface chemistry of the sorbent, but also on the physiology of the organism involved. The encouraging heavy metal removal and biomass data identified the role of both *Exiguobacterium* sp. and *Bacillus* sp as potential bioremediators, particularly in removal of chromium and nickel in alkaline pH.

Biochemical characterization of metal tolerant bacterial strains

Biochemical analysis of these strains showed that *Exiguobacterium* sp. produces diffusible pigment after 1 day of incubation, while all strains of *Bacillus* sp. produce diffusible pigment after 6 days of incubation. *Bacillus thuringiensis* strain AV2 showed amylase enzyme activity in presence of Fe, Cr and Pb. In the presence of Fe, *Bacillus aerophilus* strain IA2, *Bacillus thuringiensis* strain AV2 and *Bacillus acidicola* strain CR1 gave pink red colour (after adding methyl red indicator), which indicates that these strains are acid producing. Most of the *Bacillus* sp., in presence of Pb, Ni and Fe (except strains CR1, CR2, CR3) and only *Exi-*

Table 5. Biochemical characterization determined in terms of presence or absence of the selected enzymes.[a]

Strain	Methyl red	Mobility	Gelatinase	Pectinase					Catalase	Amylase	Fluoresence	DNase	Diffusible pigment	
				Pb	Cr	Fe	Ni	Co					1 day	6 days
CC1	–	–	–	–	+	–	–	–	+	–	–	–	+	+
AV1	–	+	+	+	++	–	–	–	+	–	–	+	–	+
IA1	–	–	+	+	–	+++	–	–	+	–	–	+	–	+
CE1	–	+	–	–	+++	+	–	–	–	–	–	+	+	+
AS1	–	+	+	+	+	+++	–	–	–	–	–	+	–	+
AS2	–	+	+	+	+	+++	–	–	–	–	–	+	–	+
CC2	–	–	+	–	+++	–	–	–	+	–	–	–	+	+
IA2	+	+	+	+	+++	+	–	–	–	–	–	+	–	+
AV2	+	+	+	+	+++	–	–	–	+	+	–	+	–	+
CE2	–	–	–	–	+++	–	–	+	+	–	–	–	+	+
CR1	+	+	+	+	++	+++	–	–	+	–	–	–	–	+
CR2	–	–	+	+	+	+++	–	–	–	–	–	–	–	+
CR3	–	–	+	+	++	–	–	–	–	–	–	+	–	+

[a] The tests for gelatinase, amylase and DNase were conducted using standard protocols. Presence of catalase was checked by the observance of effervescence on addition of H_2O_2 to isolated single colonies grown on LB medium. The pectinase–positive property was checked in presence of different metal at MIC. "+" denotes the enzyme secreting property of the isolate, whereas "–" stands for the absence of enzyme secreting property.

Table 6. Antibiotic sensitivity (μg/mL) of the nine isolates using Muller Hinton Agar.[a]

Strain	Ampicillin 10 μg	Tetracycline 30 μg	Doxycyclin 30 μg	Norfloxacin 10 μg	Cefotaxim 30 μg	Trimethorpin 30 μg	Rifampicin 15 μg	Chloramphenicol 30 μg	Metronidazole 4 μg
CC1	R	R	R	R	I	S	R	R	S
AV1	R	S	I	R	R	R	S	I	R
IA1	R	I	I	R	S	S	S	S	S
CE1	S	S	I	S	R	S	S	S	R
AS1	R	R	R	R	R	I	S	R	R
AS2	R	R	R	R	S	S	R	S	I
CC2	R	R	R	I	S	R	S	I	R
IA2	I	I	R	R	S	R	R	S	R
AV2	R	R	R	R	R	S	I	S	R
CE2	R	R	R	R	S	S	I	R	I
CR1	R	S	S	R	S	R	S	S	I
CR2	R	R	I	I	R	R	S	S	S
CR3	S	S	I	R	R	S	I	S	R

[a] The concentration of the antibiotics is in μg per disc. R denotes resistant, I denotes intermediate response, and S denotes sensitive.

guobacterium aestuarii strain CE1 (in presence of Cr) secrete extracellular DNase. In accordance with Crapart et al. (2007) almost all the *Exiguobacterium* sp. exhibit positive catalase activity, which indicates their defence mechanism against the reactive oxygen species. However, *Exiguobacterium aestuarii* strain CE1 did not show catalase activity. Pb, Cr, Fe tolerant *Bacillus* strains and Co tolerant *Exiguobacterium* strain CE2, showed a clear zone around the colonies in plates of minimal media supplemented with 1% pectin flowed with cetrimide solution after four days of incubation indicating the presence of pectinase (Table 5).

Several reports indicate a correlation between antibiotic resistance and metal tolerance (Spain 2003). In certain cases metal tolerance mechanism contribute to the increase in antibiotic resistance; the occurrence of this phenomenon can be attributed to the clustering of these genes in the same plasmid. The antibiotic sensitivity of the identified bacterial strains was tested to explore this correlation. Eleven of the thirteen isolates were moderately to highly tolerant towards selective antibiotics. The bacterial isolates of *Bacillus* strain IA1 and *Exiguobacterium* strain CR1 were sensitive to most of them (Table 6). The antibiotic sensitivity profile of these isolates can provide serious insights to explore the molecular mechanisms of resistance and correlation between metal and antibiotic resistance.

Growth profile analysis

Growth profile study is important while characterizing a bacterial species for its application in bioremediation. The growth profile of all strains followed a sigmoid pattern.

When evaluating the effect of rhizobacteria on phytoremediation in contaminated soil, regardless of the precise effects used by the bacterium to protect plants, literature reports suggest that certain bacteria may eventually find a use in the development of bioremediation strategies. The interface between microbes and plant root (rhizosphere) may have a great influence on both the increase of nutrient uptake and decrease of metal toxicity (Smith 1994). The amelioration of heavy metal toxicity in plants by microorganisms may be through reduction in the metal uptake by plants (Vivas et al. 2006) or through reduction in the amounts of detrimental stress ethylene induced by heavy metals with no effect on their uptake (Burd et al. 1998; Rajkumar et al. 2006). On the other hand, metal resistant microbes with plant growth promoting characteristics could improve the availability of heavy metals for plant uptake through solubilization or mobilization, and can be effectively utilized for phytoremediation (Abou-Shanab et al. 2003). Our study suggests that *Bacillus weihenstephanensis* strain IA1 and *Exiguobacterium aestuarii* strain CE1 with selective antibiotic sensitivity along with high Ni^{2+} and Cr^{6+} removal capabilities, respectively, can be prospective candidates for bioremediation. In addition to their role in bioremediation, characterization of these thirteen bacterial species led to the identification of some enzyme producing microbes as well. Assigning positive role of these species as amylase, pectinase or catalase producers can also be explored.

With the available knowledge it is still difficult to clarify specific features of microbial-plant and microorganism-soil interactions in the rhizosphere. Further research is thus required to understand the mechanisms involved in mobilization and transfer of metals in order to develop future strategies and optimize the bioremediation process.

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