Environ Sci Pollut Res (2017) 24:9723–9733
DOI 10.1007/s11356-017-8624-2

RESEARCH ARTICLE

Copper-tolerant rhizosphere bacteria—characterization and assessment of plant growth promoting factors

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Received: 4 February 2016 / Accepted: 9 February 2017 / Published online: 1 March 2017

Abstract Remediation of heavy metal contaminated soil is a major problem or concern worldwide. Heavy metal accumulation in the soil is increasing day by day by industries, mines, agriculture, fuel combustion and municipal waste discharge. Such contaminated soils harbour a large number of resistant microbial populations. Screening and isolation of such microbes would be utilized for natural remediation of metal contaminated soils. Therefore, in the present study, highly copper-tolerant bacteria from rhizosphere soil of *Cynodon dactylon* grown in brass effluent contaminated soil were isolated and assessed for plant growth promoting factors. A total of 61 isolates were isolated from the rhizosphere of three contaminated sites. Six highly copper-tolerant isolates named as MYS1, MYS2, MYS3, MYS4, MYS5 and MYS6 were isolated through enrichment in copper containing nutrient broth. 16S rRNA analysis revealed that the isolates were from genera *Stenotrophomonas* and *Brevundimonas* and belong to classes *Alpha Proteobacteriacea* and *Gamma Proteobacteriacea*, respectively. Strain MYS1, MYS2 and MYS4 showed 95–99% similarity with *Stenotrophomonas acidaminiphila*, strain MYS3 and MYS5 showed 99 and 97% similarity with *Stenotrophomonas maltophilia* and *Stenotrophomonas* sp. Strain MYS6 showed 94% similarity with *Brevundimonas diminuta*. All the rhizobacteria showed plant growth promoting traits such as production of siderophores, indole acetic acid (IAA), phosphate solubilization and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. From this study, we can conclude that all the isolates possess copper resistance and potential for phytoremediation of copper polluted soils.

Keywords Rhizosphere · Copper-tolerant bacteria · 16S rRNA · Enrichment · *Stenotrophomonas* sp. · *Brevundimonas* sp. · Brass contaminated soil · Heavy metal

Introduction

Soil contamination by heavy metals is a matter of concern worldwide and is getting worse day by day. Their toxicity in plants, animals and human beings is of greater concern because of their non-biodegradable nature (Jang et al. 2006; Li et al. 2006). According to the World Health Organization (WHO 2010), lead (Pb), nickel (Ni), chromium (Cr), cadmium (Cd), cobalt (Co), copper (Cu), zinc (Zn) and mercury (Hg) are considered as priority metals that need immediate attention due to their impact on human health through the food chain. Copper is among the most widely used metals in electroplating and electrical industries. Copper is a transition heavy metal with atomic number 29 in the periodic table. From centuries, Cu is used in the treatment of various diseases and as antimicrobial agent in antiseptics, medical devices, oral hygienic products, paints and fungicides (Michels 2006). Copper is essential for living organisms at lower concentration, but toxic at higher concentrations (Gordon 1994; Cervantes and Gutierrez-Corona 1994). The normal copper concentration of unpolluted soils may range between 1.6 and 7.5 mg/kg soil (Sabry 2009). But due to mining, application of agricultural chemicals, sewage treatment processes, waste incinerators, metal-producing industries and coal-fired thermal power, plant copper concentration is found to increase above permissible limits in contaminated soils.

Responsible editor: Philippe Garrigues

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Excessive copper in soil is toxic to most of the plants and soil microbes (Xu et al. 2006; Gang et al. 2013). Roots absorb copper ions from the soil and translocate to other parts of the plant (Song et al. 2004), by which host plant's metabolic and developmental physiology gets impaired. In a similar way, higher copper concentrations modify the soil microbial communities and affect the soil health by decreasing microbial population and activity (Kozdroj et al. 1995; Kim et al. 1996). It also increases the microbial resistance to copper (Atlas and Bartha 1998). Exploring this resistant microbial population from contaminated soils will provide new insights in natural remediating processes.

Biological remediation methods have drawn much attention throughout the world as they are ecologically safe and economical for the re-establishment and remediation of polluted soils. A number of studies are available on phytoremediation of polluted sites by metal accumulating plants (Belimov et al. 2005; Chehregani and Malayeri 2007; Ma et al. 2015; Dhiman et al. 2016). To understand the mechanism of mutually benefit relationships between rhizobacteria and plants is an area of interest among researchers. The rhizosphere provides biologically active microenvironment, where microorganisms form unique communities in association with plant roots. Here, the microorganisms produce and release compounds which nullify or lower the harmful effects of metals and other contaminants on plants and in return plant roots releases some exudations, which could be utilized by microbes as energy source. In contaminated rhizosphere, bacteria tend to acquire tolerance against the contaminant in a long course of time. These metal-tolerant rhizobacteria having plant growth promoting factors could be used for remediation of contaminated soils. Plant growth promoting rhizobacteria (PGPR) are known to exhibit different mechanisms such as phosphate solubilization, biological nitrogen fixation, siderophore secretion, 1-Aminocyclopropane-1-carboxylate deaminase (ACC) release, phytohormone production and promoting beneficial plant-microbe symbioses (Zhuang et al. 2007; Sharma et al. 2005, 2008) to combat stressed environment.

Many studies have been done on bacterial profiling in metal-polluted surface soils but studies on rhizobacterial population of plants grown in heavy metal polluted soils such as copper-contaminated soils are limited. Studies on rhizobacterial population from contaminated soils could add more information to our knowledge regarding diversity and metal-tolerance mechanisms. This study was conducted in search of highly copper-resistant indigenous rhizobacteria from brass industry effluent contaminated site. Rhizosphere soils associated with *Cynodon dactylon* were collected from three contaminated sites. An attempt was made to isolate and characterize rhizosphere bacteria. A total of 61 isolates were enumerated from rhizosphere soil which was further enriched with increased copper concentration to obtain highly copper-tolerant isolates. The isolated copper-tolerant rhizobacteria were characterized employing 16S rRNA sequencing method. The plant growth promoting potential of the isolated strains was also determined.

Materials and methods

Study area

The study area Moradabad, known as 'Brass city' in India is located between 28°21′ to 28°16′ N latitude and 78°4′ to 79° E longitude (Fig. 1). It covers a geographical area of 3493 km^2 with more than 400 brass and electroplating industries. The study area is drained by a small stream 'Karula' ending in Ramganga, a tributary of river Ganga. The effluents from the brass and electroplating industries are drained into the Karula stream. The site was habituated mainly with grasses (*C. dactylon*). *C. dactylon* can grow in adverse environmental conditions such as salinity, drought and contaminated soils. It is well known for heavy metal bioaccumulation and stabilization. The density of vegetation increases along the downstream of the drain as shown in Fig. 2.

Rhizosphere soil sampling and characterization

Field study was carried out along the downstream of Karula drain, Moradabad (India). The sampling of rhizosphere soil (soil around the roots) was done from three selected sites situated approximately at equal distance along the course of the Karula Nala drain. Sampling was done with wooden spatula. Soil at each site was dug at 15–30 cm depth. Sampling site 1 was at the upper end of the stream, site 2 and 3 at the middle and lower end of the stream, respectively. Random mixed sampling method was followed for each site. From each site, five healthy metal-tolerant *C. dactylon* with their roots were uprooted and the soil firmly adheres to the roots was obtained by brushing. The rhizospheric soil obtained from the roots of the plants from each site were mixed together to get three composite samples. The samples were put in sterile polythene bags, carried to the laboratory at 4 °C and analysed for heavy metals and other physicochemical parameters. Sieved (2 mm sieve) and room-dried soil samples were characterized for pH and electrical conductivity at room temperature by electrode method, percent organic carbon and organic matter by Walkley and Black (1934) method, available nitrogen by Kjeldahl method (Bremner 1960) and available phosphorous by Olsen et al. (1954) method, respectively. Copper, zinc, chromium, cadmium and nickel were extracted in HNO_3:H_2SO_4:$HClO_4$ in ratio of 5:1:1 and analysed by inductively coupled plasma optical emission spectrometry (Perkin Elmer Optima 5300 DV ICP-OES). All the chemicals and reagents used were of AR grade.

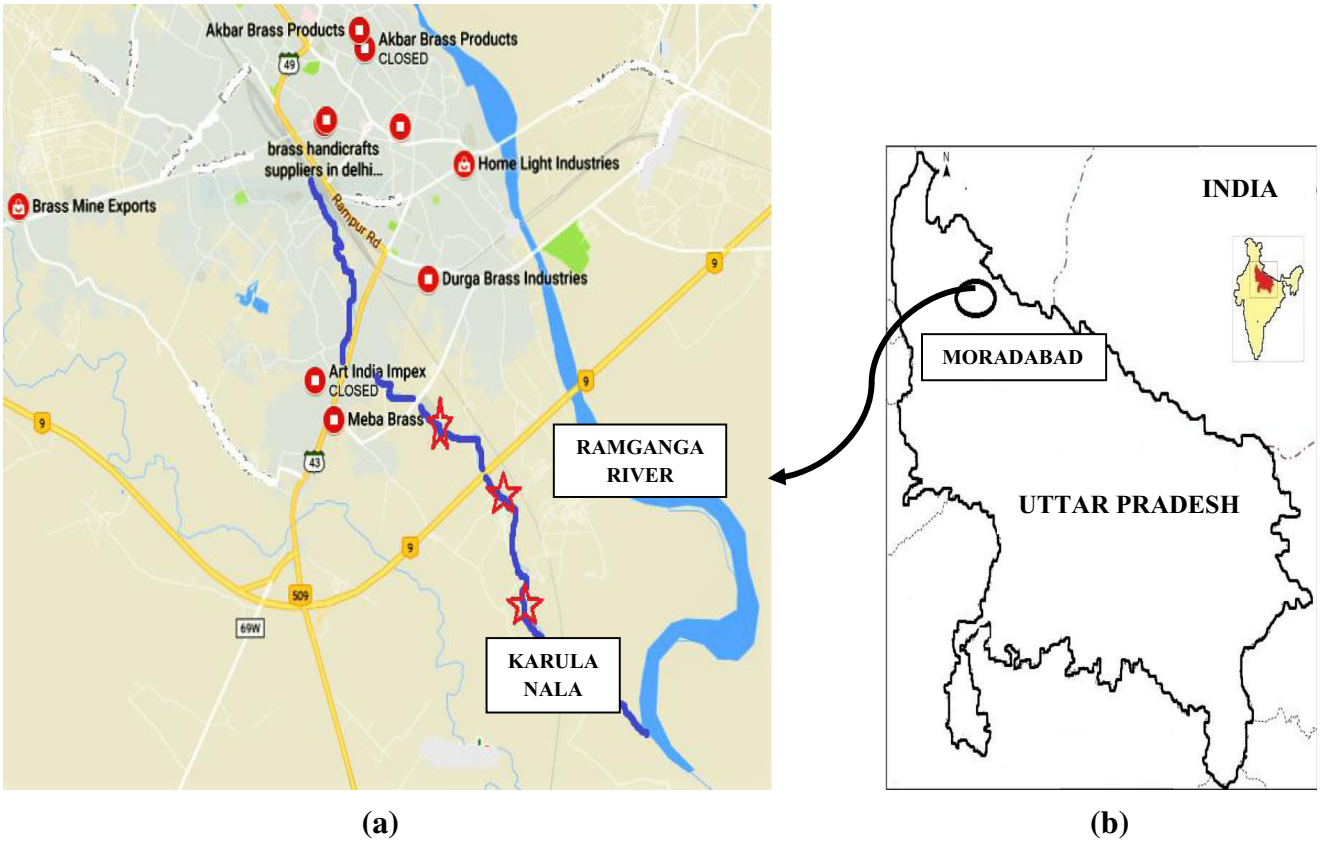

Fig. 1 Map showing **a** sampling sites (*empty star*) and **b** Moradabad (India) region

Isolation and enumeration of copper-tolerant rhizobacteria

Soil suspension was made from 1 g rhizosphere soil in 50 mL of 0.9% sterile saline water. After shaking at 150 rpm for 10 min, 100 µl of tenfold serially diluted soil suspension were plated onto nutrient agar medium (pH 7.0) to determine the total cultivable bacteria. The media was supplemented with cycloheximide to inhibit fungal development. Petri plates were incubated at 30 °C for 7 days and total isolates from three sites were enumerated.

Enrichment and isolation of copper-tolerant rhizobacteria

Enrichment of copper-tolerant rhizosphere bacterial consortium was done in Erlenmeyer flasks containing 100 mL nutrient broth (5 g of Peptone, 1 g of Beef extract, 2 g Yeast extract and 5 g NaCl in 1 L distilled water) and copper sulphate ($CuSO_4.5H_2O$) in increasing concentration from 50 to 600 mg/L. At higher Cu concentration, there was decrease in bacterial growth. Therefore, enrichment was done in broth containing copper up to 600 mg/L. The pH of the broth was maintained at 7. Three millilitres of inoculum was added to the broth and kept for incubation in incubator shaker at 30 °C and

Site 1 Site 2 Site 3

Fig. 2 Karula drain showing profuse growth of *Cynodon dactylon*, native grass

120 rpm for 5 days. The enrichment cycle was repeated three times for each copper concentration. After enrichment, copper-resistant bacteria were isolated by plating. Six morphologically different pure isolates were obtained by repeated streaking on nutrient agar medium. Colony morphology (colour, size, shape, opacity, margins) and gram staining was studied for the six different isolates.

Rhizobacterial 16S rRNA gene sequence analysis

Isolated pure culture of copper-tolerant rhizobacterial strains in glycerol stock were sent to Macrogen for16S ribosomal RNA gene sequence analysis. Briefly, pure colonies were suspended in 0.5 mL of saline water and centrifuged at 10,000 rpm for 10 min. Pellet was resuspended in InstaGene matrix and put for incubation. Incubated suspension was heated at 100 °C for 10 min. After heating, 1 μl supernatant was used for polymerase chain reaction in 20 μL of reaction mixture. Amplification of gene was carried out by using: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACG GYTACCTTGTTACGACTT-3′) universal primers. Thermal cycler was programmed to carry out 35 cycles at 94 °C for 45 s, 55 °C for 60 s and 72 °C for 60 s. Purification of PCR products was done by Montage PCR Clean up kit (Millipore). And finally, the PCR products were partial sequenced by using primers: 518F (5′-CCAGCAGCCGCGGTA ATACG-3′) and 800R (5′-TACCAGGGT ATCTAATCC-3′).

Phylogenetic analysis

Phylogenetic analysis was done as explained by Feris et al. (2003). 16S rRNA gene sequences of rhizobacteria obtained in the study were checked by Decipher (version 1.14.5) to exclude chimera sequences. Obtained forward and reverse sequences were aligned by Codoncode aligner. Aligned sequences were uploaded in BLAST (Basic Local Alignment search tool) from the website of NCBI (National Centre for Biotechnology Information) for comparison of most homologous sequences in database. Sequences with high query cover and identity percentage were considered. The sequences were submitted to NCBI through Bankit and Sequin for accession number. Phylogenetic tree construction was done with MEGA6 software by selecting maximum likelihood algorithm option in the software and Tamura 3-parameter model was selected. The sequences obtained from the study had been deposited in the GenBank database under accession numbers KJ464995 and KJ664227-KJ664231.

Characterization of isolates for plant growth promoting factors

Copper-resistant rhizobacteria isolates were tested for different PGP factors such as production of siderophore, indole acetic acid (IAA), ACC deaminase and phosphate solubilization.

Production of siderophores

Siderophore production by isolates was determined by Schwyn and Neilands (1987) method. Agar plates containing Chrome azurol S (CAS) dye were inoculated with isolated bacteria and incubated at 30 °C for 72 h. Siderophore production is indicated by the ability of the bacteria to convert blue colour medium into yellow halos around the colonies.

Indole acetic acid production

Production of indole acetic acid in isolated rhizobacteria was determined by Gordon and Weber (1951) method. Twenty millilitres of nutrient broth containing 0.5 mg/mL tryptophan was inoculated with rhizobacteria and incubated for 4 days at 30 °C and 120 rpm. After incubation, 1 mL culture was mixed with 2 mL of Salkowski's reagent and kept at room temperature for 20 min. IAA production was indicated by the development of pink colour. Absorbance was measured at 535 nm spectrophotometrically.

Utilization of ACC as nitrogen source

Activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase was estimated by evaluating the utilizing capacity of rhizobacterial isolates to ACC as main source of nitrogen. The isolated strains were inoculated in 50 mL DF (Dowrkin and Foster 1958) salt minimal medium containing 5 mM ACC. Medium was incubated at 30 °C and 120 rpm. The rhizobacterial growth was determined by taking optical density at 550 nm on the third day of inoculation. The strains were categorized as high (OD_{550} >0.7), medium (OD_{550} 0.5–0.69) and low (OD_{550} <0.5) deaminase activity on the basis of the optical density. An un-inoculated flask was maintained as control.

Phosphate solubilization

Bacterial isolates were platted on modified Pikovaskaya medium which contained dextrose 10 g, yeast extract 0.5 g, calcium phosphate 5 g, potassium chloride 0.2 g, ammonium sulphate 0.5 g, magnesium sulphate 0.1 g, manganese sulphate 0.0001 g, ferrous sulphate 0.0001 g and agar 16 g per litre (Pikovaskaya 1948) as explained by Gupta et al. (1994). Plates were incubated for 5 days at 30 °C. Production of clear zone around bacterial colonies indicated phosphate solubilizing property. Organic acid secretion was detected by production of yellowish colour zone instead of clear zone. By measuring colony and halo zone diameter,

Table 1 Physico-chemical characterization of rhizosphere soil from three sites

Soil parameters	Site 1	Site 2	Site 3
pH	7.69 ± 0.04*	7.56 ± 0.01**	6.97 ± 0.25*
Electrical conductivity (mS)	2.12 ± 0.14*	3.63 ± 0.04**	15.2 ± 0.1*
Organic carbon (%)	0.195 ± 0.14*	0.345 ± 0.057*	3.9 ± 0.3*
Organic matter (%)	0.34 ± 0.14*	0.594 ± 0.057*	6.72 ± 0.3*
Available nitrogen (mg/kg)	184.5 ± 0.03*	193.3 ± 0.05*	281.2 ± 0.2*
Available phosphorous (mg/kg)	293.3 ± 0.01*	295.3 ± 0.01*	363.7 ± 0.7*

Values are the mean values of triplicates ±standard deviation, significant at $p < 0.05$

*Significant

**Non-significant

phosphate solubilization index (SI) (Premono et al. 1996) was calculated by the formula:

$$\mathrm{SI} = \frac{\text{Colony diameter} + \text{halo zone diameter}}{\text{Colony diameter}}$$

Statistical analysis

All the experiments were carried out in triplicates and statistical calculations such as mean was done with Microsoft Office Excel 2007. Codon Code Aligner, Sequin and MEGA 6 software were used for alignment, sequence submission and phylogenetic tree construction. To analyse the effects of copper concentration on the rhizobacteria diversity, the Shannon diversity index was used and calculated from different type of colonies obtained before and after copper enrichment as follows:

$$H = -\sum_{i=1}^{s} (pi \times \log pi)$$

Where $\sum$ is summation, S is colony richness and *Pi* is the ratio of total number of different colonies obtained to the total number of samples.

Relative abundance (%) was calculated to know the percentage of a single bacterial species to the total number of copper-tolerant bacterial species obtained after enrichment. Relative abundance (%) of copper-tolerant rhizobacteria was calculated as follows:

$$\text{Relative abundance } (\%) = \frac{\text{Number of single bacterial species} \times 100}{\text{Total number of bacteria}}$$

Results and discussion

Rhizosphere soil characterization

Soil physico-chemical characterization

The characterization of rhizosphere soil collected from Brass industry effluent contaminated sites along Karula Nala (drain) is summarized in Table 1. The soil was dark grey coloured and gave off pungent smell. Soil from site 1 and 2 was slightly alkaline (7.69, 7.56) while site 3 showed neutral to acidic pH (6.97). This might be due to presence of copper in higher concentration as reported by Ssenku et al. (2014). Higher copper imparts acidic nature to the soil. Soil pH governs the availability of nutrients and toxicity of contaminants to the plant. The electrical conductivity (EC) ranged from (mS/cm) 2.12 to 15.2. EC for site 3 was much higher than that of site1 and 2; this might be due to presence of more soluble metal ions. The total organic carbon (%) ranged from 0.195 to 3.9 indicating the presence of some organic matter and compostable substances in effluent discharged (Mumoz et al. 1994; Oviasogie and Omoruyi 2007). The total organic carbon results obtained are lower than those reported by Berg et al. (2012). Correspondingly, the total organic matter (%) also varied in the range of 0.34 to 6.72. The total organic carbon (%) and total organic matter (%) obtained in the present study are much higher than that reported by Mathur and Kumar (2013). Soil organic carbon could be utilized by the soil microbes as a source of energy. The soil organic matter influences the physico-chemical processes of the soil and is an important indicator of root environment (Okalebo et al. 1993). Available phosphorus and available nitrogen are the most important nutrients required for proper growth, respiration and reproduction of plants. Available phosphorus and available nitrogen ranged from 293.3 to 363.7 mg/kg and 184.5 to 281.2 mg/kg, respectively. Higher percent organic

Table 2 Metal concentration in three rhizosphere soil samples

Metal	Site 1	Site 2	Site 3	MPL[a] for soils (mg/kg)
Copper (mg/kg)	573.3	784.1	1498	100
Zinc (mg/kg)	679.3	934.6	1533	300
Cadmium (mg/kg)	0.8	0.9	1.7	0.6
Chromium (mg/kg)	71.9	105.9	183.3	250
Nickel (mg/kg)	106.8	149	297.7	60

Values are significant at $p < 0.05$

[a] Maximum permissible limit (SEEPA 1995)

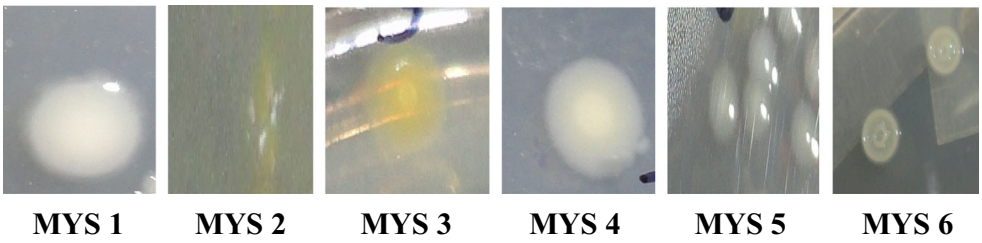

Fig. 3 Isolated pure rhizobacterial colonies after enrichment

matter is the possible reason for increased organic carbon (%), available nitrogen and phosphorous of the soil. Results of all physicochemical parameters show the highest values in site 3 and the lowest values in site1. Location of site 3 on the lower course of the drain is the reason for increased concentration of pollutants in the soil.

Heavy metal analysed

The results of heavy metal analysis revealed that the concentrations of all the metals studied were significantly higher than the maximum prescribed limits for soil (Table 2). Copper in soil samples ranged from (mg/kg) 573.3 to 1498 with an average concentration of 951.8 mg/kg. Organic matter is one major factor that determines the retention of Cu in the soil, irrespective of pH (Parth et al. 2011). Zinc concentration varied from (mg/kg) 679.3 to 1533 with average concentration of 1049 mg/kg. Under alkaline conditions, Zn is absorbed by organic matter and in acidic soils, it is one of the most soluble and mobile metal (Parth et al. 2011). The results obtained in the present study were higher than those reported by Iyaka and Kakulu (2012) from a Brass industrial area of Nigeria. They reported Cu ranged from 27 to 1286 $\mu g g^{-1}$ and Zn ranged from 114 to 236 $\mu g g^{-1}$ in topsoil. Similarly, cadmium and nickel ranged from (mg/kg) 0.8 to 1.7 and 106.8 to 297.7 with average concentration of 1.13 and 184.5 mg/kg, respectively. Chromium ranged from 71.9 to 183.3 mg/kg and is below the maximum prescribed limit (250 mg/kg). Nickel and chromium levels recorded in this study are elevated; Singh et al. (2016) reported 72–243 and 21.5–47 mg/kg in electroplating industrial effluent. Increased levels of copper and zinc may be attributed to their significant use as the major metals in brass industries. Presence of cadmium, nickel and chromium might be due to the effluent discharge from some electroplating industries located in that region. Heavy metals followed a gradient from site 1 to 3. Site 3 showed higher concentration due to its location at the downstream of the drain. The water flow at downstream is very slow that might have led to more accumulation of metals in the soil.

Enumeration, enrichment and isolation of copper-tolerant rhizosphere bacteria

A total of 61 (22, 18, 21) cultivable rhizobacterial isolates were obtained from the rhizosphere soil samples collected at three different contaminated sites. The colonies grown on nutrient media were differentiated on the basis of their colony morphology such as size, shape, colour, margin surface, elevation and opacity. The consortium was further enriched with increasing concentration of copper (up to 600 ppm). Enrichment cycles resulted in six rhizobacterial isolates (Fig. 3) showing tolerance to copper. Copper tolerance in these isolates is higher than reported in previous studies (Shakoori and Muneer 2001; Zaki and Farang 2010; Opulencia et al. 2015). Pure colonies were obtained after repeated streaking in nutrient agar. Isolated rhizobacteria showed different morphological appearance as summarized in Table 3. All the six isolates were identified to be gram-negative bacilli with white, off-white and yellow colour colonies with diameter ranging between 0.2 and 0.7 cm. Most of the colonies were predominantly circular in form.

Shannon diversity index (H) was calculated to determine the effect of copper on diversity of Cu- tolerant rhizobacteria. The Shannon diversity index before and after Cu enrichment

Table 3 Colony morphological characteristics of the rhizobacteria isolated from contaminated soil

Strain	Form	Size (cm)	Colour	Edge	Surface	Aspect	Consistence	Elevation	Opacity
MYS1	Circular	0.4	White	Entire	Concentric	Wet	Soft	Raised	Translucent
MYS2	Circular	0.2	Yellow	Entire	Smooth	Wet	Soft	Convex	Translucent
MYS3	Circular	0.4	Yellow	Entire	Concentric	Wet	Soft	Convex	Translucent
MYS4	Circular	0.6	White	Entire	Concentric	Wet	Soft	Flat	Translucent
MYS5	Circular	0.2	Off-white	Entire	Smooth	Wet	Soft	Convex	Translucent
MYS6	Irregular	0.7	Off-white	Undulate	Smooth	Wet	Soft	Raised	Opaque

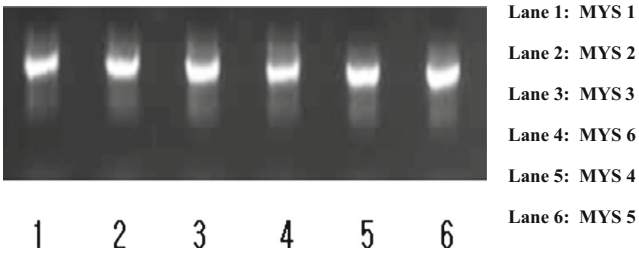

Fig. 4 Gel bands of 16S rRNA genes from the six metal-tolerant isolates

were 1.09 and 1.08, respectively. A reduction in Cu-tolerant bacteria was observed after enrichment in higher Cu concentration.

16S rRNA analysis

PCR amplification reactions produced 16S ribosomal RNA gene amplicons of approximately 1400 bp. Figure 4 shows the separation pattern of six PCR-amplified samples on agar gel electrophoresis.

The sequences were aligned, checked for chimera sequences and the most similar match was identified by BLAST search. 16S rRNA gene sequencing revealed that five isolates among six belonged to genera *Stenotrophomonas* with variation at species level. Strains MYS1 and 4, MYS2 showed 99 and 95% sequence identity with *Stenotrophomonas acidaminiphila*. Strain MYS3 and MYS5 showed 99and 97% sequence identity with *Stenotrophomonas maltophilia* and *Stenotrophomonas* sp., respectively. *Brevundimonas* was the only different genus represented in the consortium and it showed 94% sequence identity with *Brevundimonas diminuta*. The occurrence of genus *Stenotrophomonas* along with other rhizobacteria in copper-contaminated site is reported in previous studies (Andreazza et al. 2010; Ghosh et al. 2011; Altimira et al. 2012; Ghosh and Saha 2013). Indeed, genus *Brevundimonas* has been reported in soils contaminated by metals (Dell'Amico et al. 2008; Cavalca et al. 2010; Xiao et al. 2010) (Table 4).

Figure 5 provides the relative abundance of Cu-tolerant rhizobacteria. *Stenotrophomonas acidaminiphila* was dominant species representing about 50% of the rhizobacterial species. *S. maltophila*, *Stenotrophomonas* sp. and *B. diminuta* each represented 16.7% of the total species.

Phylogenetic analysis of 16S rRNA

In phylogenetic analysis (Fig. 6), strain MYS6 was totally different from rest of the strains. Strains MYS1, MYS2, MYS3, MYS4 and MYS5 appeared indistinguishable and clustered with members of genus *Stenotrophomonas*. Among them, strain MYS1, MYS4 and MYS5 are closely related while MYS2 showed difference from the rest of the strains.

PGPF analysis

Plant growth promoting (PGP) rhizobacteria is reported to increase the biomass and yield of the plant under biotic and abiotic stressed environment. They reduce the deleterious effect of metal stress on plants (Rathaur et al. 2012; Płociniczak et al. 2013; Singh et al. 2013). The PGP traits such as siderophore production, release of IAA, ACC deaminase activity and phosphate solubilization capability were assessed in the six isolates and are summarized in Table 5. All isolates were able to produce IAA in range of 5.4 to 7.8 μg/mL. IAA-producing rhizobacteria stimulate root cell division and cell elongation and hence promotes root growth (Glick and Penrose 1998). Zaidi et al. (2006) and Ma et al. (2009) in their study stated the importance of IAA in the establishment of plant-bacterial interaction and plant growth promotion in contaminated soils. Khalid et al. (2004) categorized rhizobacteria into three groups on the basis of in vitro production of IAA. One to 10 μg/mL producing bacteria were classified as lower, medium and high producers are those that produce 11 to 20 and 21 to 30 μg/mL IAA, respectively. All the isolates in this study could be classified as lower producers. In fact, Dell'Amico et al. (2008) observed increased production of IAA under cadmium stressed conditions. IAA in addition to providing defence to cope with stressful conditions, it enhances the uptake of metals and other elements (Fässler et al. 2010; Aksorn and Chitsomboon 2013).

Siderophores are known for the reduction of Fe^{3+} to Fe^{2+}, the form of iron which is utilized by the plants. In the same way, bacterial siderophores complexes with other metals including Pb, Mo, Ni, Cd, As, Cu, Al, Co, Ga, Zn and U (Farkas et al. 1997; Bhattacharya 2010) increases the bioavailability of metal to the plant in contaminated soil (Rajkumar et al. 2010).

Table 4 Accession numbers and percent similarity of six rhizobacterial isolates submitted in GenBank

S. No.	Strain	Percent identity (%)	Bacteria	Accession number
1.	MYS1	99	*Stenotrophomonas acidaminiphila*	KJ664227
2.	MYS2	95	*Stenotrophomonas acidaminiphila*	KJ664228
3.	MYS3	99	*Stenotrophomonas maltophilia*	KJ664229
4.	MYS4	99	*Stenotrophomonas acidaminiphila*	KJ664230
5.	MYS5	97	*Stenotrophomonas sp*	KJ664231
6.	MYS6	94	*Brevundimonas diminuta*	KJ464995

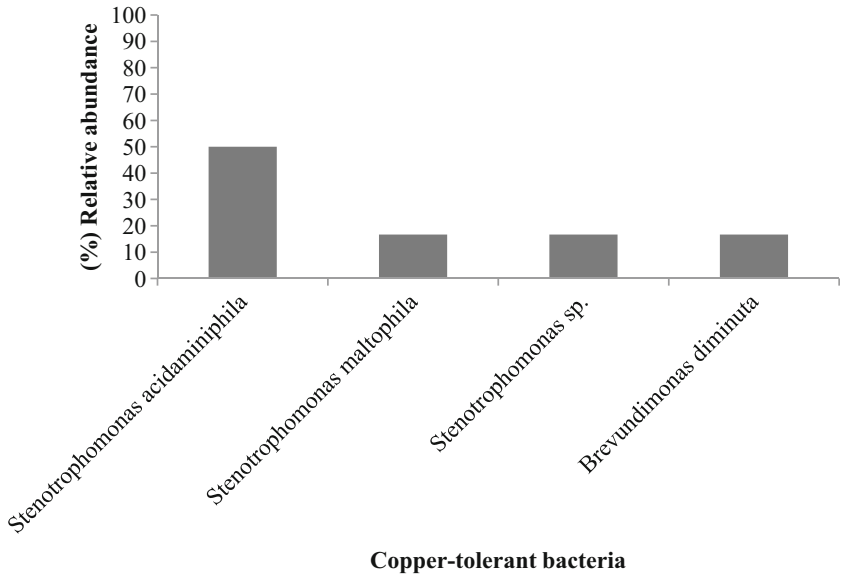

Fig. 5 Relative abundance of species from two different genera, isolated after enrichment in copper nutrient agar medium

Hence, bacterial siderophores reduce the heavy metal stress on plants and play a major role in heavy metal bioremediation. All the tested isolates were siderphore producers. The isolates were classified on the basis of production of yellow halo zone diameter: low (<0.2 cm), medium (0.2 to 0.5 cm) and high siderophores producers (>0.5 cm). MYS6 (0.38 cm) could be classified as medium siderophore producer. MYS1 (1.0 cm), MYS2 (0.63 cm), MYS3 (1.1 cm), MYS4 (0.63 cm) and MYS5 (0.76 cm) are classified as high siderophore producer. Highest siderophore production was observed in strain MYS3.

Further, the isolates were also examined for its ACC (1-aminocyclopropane-1-carboxylate) deaminase activity in terms of optical density. Results reveal that all isolates showed ACC deaminase activity. MYS2 (0.745 nm), MYS5 (0.930 nm) and MYS6 (0.717 nm) showed higher activity among the six isolates. Less ACC deaminase activity was observed in MYS3 (0.374 nm). The ACC deaminase activity of strains MYS1and MYS4 was 0.621 and 0.597 nm, respectively. Similarly, Zhang et al. (2011) reported higher ACC deaminase activity (213 to 370 μM α-ketobutyrate/mg h) in copper-tolerant endophytic bacteria. ACC is utilized as main

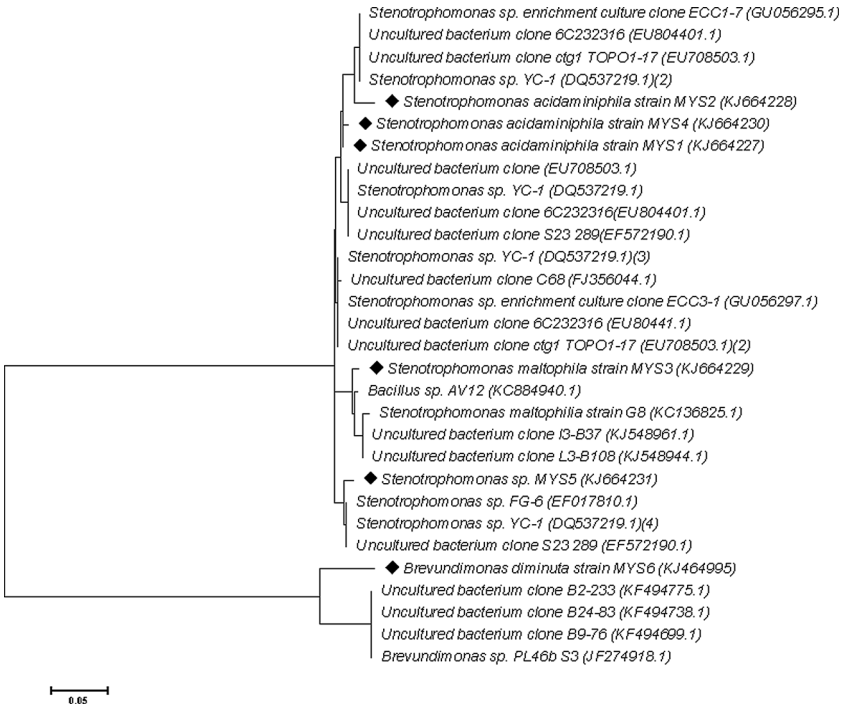

Fig. 6 Phylogenetic tree of copper-tolerant rhizobacteria and related sequences from identified bacteria in database. The *bar* represents 0.06 substitutions per site

Table 5 Plant growth promoting factors in copper-tolerant rhizobacteria

Strain	IAA (μg/mL)	Siderophore (cm)	ACC deaminase (nm)	P solubilization index (PSI)
MYS1	7.8 ± 0.3	1.0 ± 0.01	0.621 ± 0.003	ND
MYS2	6.7 ± 0.2	0.63 ± 0.05	0.745 ± 0.005	2.5 ± 0.18
MYS3	5.4 ± 0.1	1.1 ± 0.06	0.374 ± 0.004	2.6 ± 0.10
MYS4	6.0 ± 0.12	0.63 ± 0.03	0.597 ± 0.006	2.2 ± 0.13
MYS5	6.8 ± 0.3	0.76 ± 0.05	0.930 ± 0.002	3.2 ± 0.10
MYS6	7.1 ± 0.2	0.38 ± 0.07	0.717 ± 0.001	3.8 ± 0.20

Each value is a mean of the triplicate experiment ±standard deviation

ND not detected

source of nitrogen to regulate the ethylene biosynthesis in roots. This results in formation of longer and denser roots in contaminated sites. ACC deaminase producing bacteria plays an important role in improving plant growth under adverse environmental conditions especially in hyper-accumulators. Belimov et al. (2005) in his study reported that ACC promoted root and shoot growth in Indian mustard and rape (*Brassica napus*) seedlings when subjected to cadmium chloride ($CdCl_2$) solution. The ACC is also reported to protect the plant from the toxicity of heavy metals (Burd et al.1998).

Similarly, phosphate solubilization activity was detected. Among the six isolates, all exhibited phosphate solubilization activity except strain MYS1. Very prominent clear zones extending from the edge of the bacterial colony were shown by strain MYS2, MYS5 and MYS6. Phosphate solubilization index (SI) of isolated strains ranged from 2.2 to 3.8 (Table 5). Maximum phosphate solubilization activity was showed by strain MYS6 which have phosphate solubilization index of 3.8. The results obtained are comparable with results of Mujahid et al. (2014). The solubilization of the insoluble mineral phosphate complexes increases the availability of inorganic phosphate content at heavy metal contaminated sites. This improves plant nutrition resulting in increased plant growth. Both organic and non-organic acid mediated phosphate solubilization was examined for the isolates. MYS2, MYS5 and MYS6 showed acid mediated phosphate solubilization (Fig. 7) while MYS3 and MYS4 showed non-acid mediated phenomena. Bolan et al. (1994) stated two mechanisms through which the organic acids raise the availability of phosphorous in soils: one by declining the phosphorus adsorption and other by rising phosphate solubilization.

Metal-tolerance and PGP factors are the key pre-requisite characteristics for the bacteria being used in phytoremediation of metal-contaminated soils. Any bacteria with a coupled potential of pollutant detoxification and plant growth promoting traits would have a promising role in phytoremediation of contaminated soils (Huang et al. 2004; Ma et al. 2009). Therefore, it is essential to analyse these characters in an indigenous bacterial population before developing any efficient bioremediation measures for contaminated soil.

Conclusions

The study site is heavily contaminated with heavy metals from brass industry effluent. Rhizosphere of *C. dactylon* grown in the contaminated site harbours a good number of rhizobacteria. Highly copper-tolerant rhizobacteria isolated from the contaminated site were gram-negative bacilli and belonged to genera *Stenotrophomonas* and *Brevundimonas*. All the six isolates showed in vitro excellent PGP activities such as phosphate solubilization, siderophore production and good ACC deaminase activity. Heavy metal tolerance and PGP potential assessment is the primary step in bioremediation of heavy metal contaminated sites. These results revealed that isolated copper-tolerant bacteria have potential for bioremediation. Therefore, further evaluation of the isolates with suitable plant is needed to reveal their efficiency as a good PGPR.

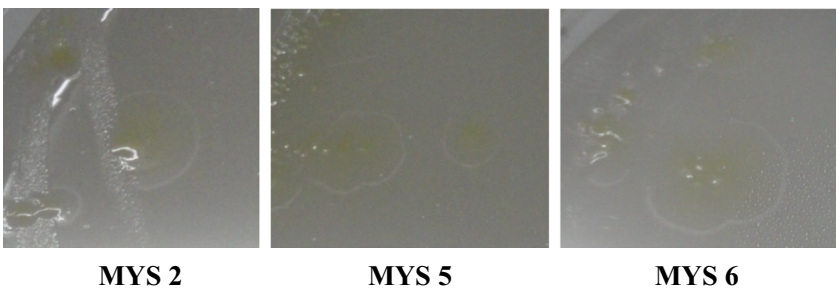

Fig. 7 Organic acid mediated phosphate solubilization by isolated rhizobacteria (source http://maps.google.com, www.mapsofindia.com), not to scale

Acknowledgements The authors wish to acknowledge the Department of Science and Technology and Sophisticated Analytical Instrument Facility of IIT Madras for their support in metal analysis.

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