RESEARCH ARTICLE

Pseudomonas: A Major Bacteria in Heavy Metal Contaminated Soil of South-West Punjab, India

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Abstract

Soil microflora is continuously changing with altered soil conditions. These soil alterations are a consequence of heavy metals entering and affecting every sphere of life. Heavy metals are not only hazardous for crops but also affect the soil microbial community. Soil bacteria with the potential of plant growth promotion and multiple metal resistances can be an instrument for crop improvement and heavy metal detoxification. In this study, predominant bacterial community associated with the heavy metal contaminated soil was studied using 16S rRNA gene sequencing in association with culture-based techniques. Elemental metal analysis of collected soil samples showed an elevated level of metal content in the soil. 16S rRNA gene analysis and phylogenetic analysis of 126 bacterial clones revealed the probable predominance of *Pseudomonas* (40.48%) followed by *Flavisolibacter* (13.49%). Based on morphological and biochemical characterization, nine *Pseudomonas* strains were selected from the soil and were further confirmed by 16S rRNA gene sequencing with 92%-100% similarity with *Pseudomonas* species. The minimum inhibitory concentration (MIC) and maximum tolerance capacity (MTC) of three essential metals Cu, Zn, and Fe were determined individually and in combinations. It was found that Zn is the most toxic metal among the three metals and the metal showed a synergistic effect in inhibiting microbial growth when used in combinations. Presence of three metal resistant/tolerant genes *czcA, pcoA* and *copB* were also determined in the isolated *Pseudomonas* sp. by PCR. The soil in this region has high concentrations of heavy metals. The indigenous *Pseudomonas* sp. has multiple metal resistances and can be used for bioremediation of heavy metals and microbe assisted phytoremediation.

Keywords: Heavy metals, Maximum tolerance capacity, Minimum inhibitory concentration, *Pseudomonas*, Toxicity.

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Introduction

Microorganisms in the soil are necessary for maintaining a balanced ecosystem. They play a significant role in the biogeochemical cycle and detoxify or remove toxins, transform minerals, metals and decompose organic matters (Altimira *et al*., 2012). But, advancement in technology and industrialization has put a negative impact on the microflora of the soil by contaminating the soil. Heavy metals affect micro-organisms by altering biochemical nature, reducing its number and alter community structure. Heavy metals are non-degradable metal elements with a density of more than 5 g cm^{-3} (Nies, 1999). Few heavy metals, e.g., Fe, Cu, Zn, Mg, P, Ni, Co, Ca are necessary for trace amount for the functioning of metabolic pathways as they are used as co-factors in enzymes and other bio-molecules (Khan *et al*., 2008). Iron (Fe), copper (Cu) and zinc (Zn) are three most important elements for the functioning of various metabolic activities and cells have fast and unspecific transport system for the intake of these elements (Nies, 1999). Hence, these metals can easily enter the cell. Copper plays a role in maintaining the strength of blood vessels, connective tissues and also in the production of hemoglobin, and melanin, etc. (Osredkar and Sustar, 2011). Whereas, iron is essential for the living system as it is required by peroxidase, catalase, and cytochrome-*c* enzymes. Iron is also needed during the formation of hemoglobin and myoglobin. While, zinc is required for gene stability and gene expression as it plays a major role in DNA replication, DNA repair, transcription, translation, and apoptosis (Dreosti, 2001).

On the contrary, excessive levels of these trace metals are harmful to the micro-organisms, humans and other living systems (Ansari *et al*., 2011). Copper act as a catalyst in the formation of reactive oxygen species and catalyzes peroxidation of membrane lipids (Stohs and Bagchi, 1995). Free iron induces lipid peroxidation of the membrane, disrupts oxidative phosphorylation (Albretsen, 2006), and zinc acts as a potent inhibitor of the respiratory electron transport systems of bacteria and mitochondria (Nriagu, 2007). Bacterial species inhabiting in metal contaminated sites are able to tolerate high concentration of heavy metals due to the presence of heavy metal resistant/reducing genes present in plasmid or in chromosomal DNA (Piotrowska-Segeta *et al*., 2005). Various operon systems such as cop, pco, ars, czc are responsible for bacterial resistance towards heavy metals such as copper, zinc, arsenic, cadmium. cop operon (*copA, copB, copC* and *copD*) is reported in *Cupriavidus metallidurans* CH34*, Pseudomonas syringae* pv. tomato PT23 and pco operon in *E. coli* are involved in resistance against copper. *pcoA* gene encodes the multi-copper oxidase which oxidizes Cu (I) to the less toxic chemical form of Cu (II) (Djoko *et al*., 2008). In *E.coli, copA* gene encodes for P-type ATPase transporter that helps in metal efflux (Altimira *et al*., 2012). In *Sulfolobus solfataricus copA* and *copB* gene both encodes for copper-transporting ATPase (Völlmecke *et al*., 2012). Czc operon is responsible for the efflux of cobalt, zinc, and cadmium. It is an resistance nodulation division (RND) family transporter. CzcA, CzcB, and CzcC are transmembrane proteins that are used for efflux of Zn across the cytoplasmic membrane (Nies and Silver, 1995). Bacterial ferric reductases *(Fre)* are responsible for the reduction of ferric citrate and variety of siderophores (Schroder *et al*., 2003). These metal resistant bacteria can be a good candidate for the purpose of bioremediation of heavy metals from soil.

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Punjab is situated in the North-West of India and mainly relies upon agriculture. Uses of chemical fertilizers, pesticides, insecticides lead to heavy metal contamination in this area. Malwa, which occupies around 15% of Punjab's cotton cultivated area and consumes around 75% of the total pesticides used in Punjab (Mittal *et al*., 2013). In our previous metagenomic bacterial community analysis, it was revealed that majority of the clones belong to class Gamaproteo bacteria and of which *Pseudomonas* is the most predominant genera in the soil around Bathinda (M.Phil. Thesis). Therefore, the present study was aimed to selectively isolate and characterize predominant bacteria *Pseudomonas* sp. from the soil, investigate their tolerance towards biologically important heavy metals Cu, Zn and Fe, and presence of heavy metal resistance genes in their genome.

Materials and methods

Soil Sample Collection

A sampling of soil was done from three different sites of Bathinda region: Central University of Punjab (30.170N, 76.450E), agriculture land of Goniana Kalan (30.32290N, 74.92340E) and agriculture land of Jassi Pau Wali (30.108750N, 75.0910E). Central University of Punjab campus soil was an abandoned soil without any agricultural activities for more than a decade and agricultural land of Goniana Kalan and Jassi Pau Wali were two villages separated by Bathinda town where agricultural activities are carried out. At each site, soil samples from (0 to 10 cm depth) were randomly collected in triplicates from three different locations that are 100m apart from each other. The triplicate soil samples from each site collected were homogenized together and composited samples were sieved. Then the samples were stored at 4°C and further used for elemental metal analysis and isolation of bacteria.

Isolation and Characterization of *Pseudomonas* sp. from Soil Samples

Isolation of Pseudomonas sp.

To isolate heavy metal resistant *Pseudomonas* sp., 1 g soil was taken in triplicates from each soil sample, suspended in 10 mL of 0.085 mol L^{-1} NaCl and serially diluted as described by Gupta *et al.* (2012) with a few modifications. 100 µL aliquot of 10^{-2} dilution was spread plated on CTAB-*Pseudomonas* agar medium plates that contain 50 ppm of copper nitrate and 50 ppm of zinc nitrate, 50 ppm of ferric nitrate separately. Then the plates were incubated at 37°C for 48 hours.

Biochemical Characterization of the Isolated Pseudomonas sp.

Strains were characterized on the basis of their Gram's staining, colony characteristics and biochemical characteristics [Catalase test (Reiner, 2010), Oxidase test (Shields and Cathcart, 2010), Citrate test (Aneja, 2003), MR-VP test (McDevitt, 2009), Urease test (Aneja, 2003), Starch hydrolysis test (Aneja, 2003) and Triple Sugar Iron agar test (Lehman, 2005)]. *Pseudomonas stutzeri* MTCC 6717 was used as a reference strain, the isolates showing similar biochemical characters were selected. The selected strains were further grown on cetrinix supplemented *Pseudomonas* agar for selective isolation of *Pseudomonas sp*. The pure cultures grown on cetrinix supplemented *Pseudomonas* agar were selected and stored at 4°C.

Molecular Characterization and Phylogenetic Analysis of the Isolated Pseudomonas sp.

Selected strains were further identified by 16S rRNA gene sequencing. 16S rRNA gene was amplified in four fragments by using universal primers A: 27F (AGAGTTTGATCMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG), B: 515F (GTGCCAGCMGCCGCGGTAA) and 907R (CCGTCAATTCMTTTRAGTTT), C: 895F (CRCCTGGGGAGTRCRG) and 1492R (GGTTACCTTGTTACGACTT) and D: 27F and 1492R. All the internal fragments were amplified along with the whole 16S rRNA gene to ensure the amplification of the whole gene without gaps. Colony PCR was employed to target the 16S rRNA gene. The reaction mixture (20 µL) consists of 0.25 mmol L^{-1} of dNTP each, 1x Taq DNA polymerase buffer, 0.15 U Taq DNA polymerase, 2.5 mmol L^{-1} $MgCl_2$, 0.5 ng of forward and reverse primer, 2 µL bacterial cell suspension (a loop full of bacterial culture in 20 µL of milliQ water) and 9 µL nuclease-free water. Following PCR cycle conditions were used for the amplification of: Fragment A: 94°C for 10 min, followed by 35 cycles of 94°C for 1 min denaturation, 55°C for 1 min annealing and 72°C for 1:30 min of extension; and a final extension at 72°C for 8 min. Fragment B: 94°C for 10 min, followed by 35 cycles of 94°C for 1 min denaturation, 52°C for 1 min annealing and 72°C for 1:30 min of extension; and a final extension at 72°C for 8 min. Fragment C: 94°C for 10 min, followed by 35 cycles of 94°C for 1 min denaturation, 50°C for 2 min annealing and 72°C for 1:30 minutes extension; and a final extension at 72°C for 8 min. Fragment D: 94°C for 10 min followed by 94°C for 1 min denaturation, 55°C for 1 min annealing and 72°C for 1 min extension for 35 cycles and a final extension at 72°C for 10 min. The quality of the PCR products was checked on 2% agarose gel. Amplified PCR products were sequenced with ABI 3730 xL DNA Analyzer using Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA). The sequences obtained were compiled using Geneious R6 software. The compiled sequences were analyzed by comparing the sequences in the GenBank database using the BLAST algorithm tool. Phylogenetic analysis was done using MEGA 7 software.

Estimation of Bacterial Tolerance towards Metals

Isolated strains were grown on nutrient agar containing various concentrations (from 50 ppm till bacteria failed to grow) of zinc nitrate, copper nitrate, and ferric nitrate. Isolates were also grown on the agar plates that contain various combinations of the metals mentioned. Triplicate plates of each isolate with different concentration of metals were incubated at 37°C and examined at intervals for up to three days. Concentration at which bacterial colony formation is completely inhibited determines the MIC. MIC_{100} is the minimum concentration at which all the members of the selected population failed to grow (Schwarz *et al*., 2010; Yilmaz, 2003). We also determined maximum capacity (MTC) that is tolerable for bacterial growth.

Detection of Heavy Metal Resistant Genes

For the detection of heavy metal resistance genes, genomic DNA was isolated from all the isolates by the method given by Chen and Kuo (1993). The primers used for *copB* gene: (F) 5'-AGGATCAGCC GATTGGTAA-3' and (R)5'-TCCGAGGGAGAGCGTACTAA-3'; *czcA* gene: (F) 5'-CAGTAGCGGTGGGTTTCATT-3' and (R) 5'-CACATGATTGG CAACAGACC-3'); and *pcoA* gene (F) 5'-GCTTCAAGGTCAAGCAGAGC-3' and (R) 5'-CGGCCCTGGTTGTAGTAGTC-3') are designed using primer 3 software. The PCR mixture contains 1 µL DNA, 1x Taq polymerase assay buffer, dNTPs 62.5 µmol L^{-1} each, 1.25 mmol L^{-1} $MgCl_2$, 3 ng gene-specific primer, 0.075U Taq polymerase, and 9 µL nuclease-free water in a final volume of 20 µL. The amplification conditions used were 2 min at 94°C; 45 cycles of 45 sec at 94°C of denaturation, 45 s at 58°C (*copB* and *pcoA*), 57°C (*czcA*) of annealing and 1:30 min at 72°C of extension; and final extension at 72°C for 7 min.

For confirmation of detected genes, amplified PCR products were sequenced with ABI 3730 xl DNA Analyzer using Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA). The compiled sequences were analyzed by comparing the sequences in the GenBank database using the BLAST algorithm tool.

Results

Elemental Metal Analysis

The elemental metal analysis showed agricultural land has higher metal concentration than non-agricultural land (Table 1). A comparison with Hundal *et al.* (2006), has made to conclude that the concentration of cadmium has increased 5 folds. Similarly, iron, zinc, and copper concentration were increased by approximately more than 100 folds, 16 folds, and 2 folds respectively (Table 2). Other metal concentrations were also increased by 5 to 10 folds; Concentration of chromium was also found to be 100 folds higher.

Isolation and biochemical characterization of *Pseudomonas* sp.

For isolation of *Pseudomonas* spp., 62 bacterial colonies were selected on the basis of colony morphology and Gram's staining. All the isolates were Gram-negative rods. Further, on the basis of biochemical characterization (according to Bergy's manual) of these 62 isolates, they were grouped into 16 groups. Group 1 showed similar characteristic with *Pseudomonas stutzeri* MTCC 6717. Group 1 includes 21 bacterial isolates. These 21 bacterial isolates were then grown in cetrinix supplemented *Pseudomonas* agar. Out of 21, only nine isolates *viz*. CCUPAS1, CCUPAS6, CCUPAS14, CCUPAS16, JCUPAS7, GCUPAS 14, GCUPAS16, JCUPAS17 and GCUPAS20 were able to grow in the cetrinix supplemented media. The nine isolated strains further used for molecular characterization.

Molecular Characterization and Phylogenetic Analysis of Isolated *Pseudomonas* sp.

Molecular characterization of selected nine isolates was done by 16S rRNA gene sequencing. 16S rRNA gene sequencing indicated that out of the nine isolates, eight isolates have 92–100% similarity to the genus *Pseudomonas* (Table 3) and one isolate was found to have 97% similarity with Gamma proteobacterium HTB095. The sequences were deposited in the GenBank nucleotide database and accession numbers KM594399 through KM594392 have obtained. Phylogenetic analysis of these isolated bacterial strains was done (Fig. 1). According to phylogenetic tree constructed, CCUPAS14, CCUPAS6, and CCUPAS16 belong to same i.e., *Pseudomonas mendocina*. Their closest neighbors were CCUPAS1 and GCUPAS20 (*Pseudomonas* sp. RA-4 and *Pseudomonas* sp. RA-6 respectively) belongs to category *Pseudomonas* RA. JCUPAS7, JCUPAS17, and GCUPAS16 are close relative to previous categories, but, GCUPAS14 strain, *i.e*., Gammaproteobacterium is most distantly related.

Heavy Metal Tolerance Capacity of Bacterial Isolates

Heavy metal tolerance of the all the nine isolates (i.e., CCUPAS1, CCUPAS6, CCUPAS14, CCUPAS16, JCUPAS7, GCUPAS14, GCUPAS16, JCUPAS17, and GCUPAS20) were determined against most essential metals, i.e., Cu, Zn and Fe and found that *Pseudomonas* isolates showed high tolerance against these metals. But, when the isolates were exposed to a combination of these metals their tolerance capacity decreases. This might be due to the synergistic effect of the metals in inhibiting bacterial growth. Isolates show maximum tolerance to Fe and least to Zn (Table 4) but, the pattern of tolerance among the isolates varied. For determining the minimum inhibitory concentrations for isolates, MIC_{100} was determined. MIC_{100} observed for Fe, Cu and Zn were 2000, 700, and 200 ppm, respectively. Therefore, the order of toxicity of these metals towards the isolates were Zn > Cu > Fe. The maximum tolerance against Cu was shown by GCUPAS16 strain and strain CCUPAS16 showed the highest tolerance against Zn. When grown against a combination of metals, all the isolates were able to grow but strain CCUPAS1, CCUPAS14, JCUPAS17, GCUPAS20, CCUPAS6 showed the highest tolerance, thus exhibiting multiple resistances. Tolerance to high concentrations of heavy metals exhibited by bacterial strains may be due to the presence of resistance genes in their genome.

Table 1: Elemental metal analysis of soil samples collected from different sites of Bathinda

	Elemental Metal Analysis Report (mg kg^{-1})		
Metals	*Campus*	*Goniana*	*Jassi Pauwali*
Arsenic (As)	0.37*	0.325	0.325
Boron (B)	12.925	13.34*	10.77
Calcium (Ca)	3761	2307.5	4016.5*
Cadmium (Cd)	0.16	0.175*	0.125
Chromium (Cr)	6.04*	5.93	4.265
Copper (Cu)	1.285	4.455*	1.23
Iron (Fe)	3169	3261*	2373
Potassium (K)	600.85	639.6*	413.6
Magnesium (Mg)	1138.6	1204.6*	991.65
Manganese (Mn)	50.225	51.655*	38.875
Sodium (N)	127.14*	81.84	106.95
Phosphorus (P)	97.005	143.72*	105.62
Sulphur (S)	95.295*	83.27	85.105
Zinc (Zn)	24.91	36.85*	12.21

*Showed highest concentration among the soil samples collected from three different sites

Detection of Heavy Metal Resistance Genes

Heavy metal resistance genes *czcA*, *copB*, and *pcoA* were detected by PCR in the genomic DNA of isolated *Pseudomonas* strains. *czcA* and *copB* gene segment of ~230 bp and ~275 bp, respectively, were obtained by PCR in all the isolated *Pseudomonas* strains and *pcoA* gene ~220 bp in length were amplified in GCUPAS16 and CCUPAS6. These amplified products of *copB* and *czcA* from strain GCUPAS20 and *pcoA* from strain GCUPAS16 were subjected to sequencing analysis (Fig. 2). Partial sequences of *czcA*, *copB* and *pcoA* genes obtained showed 100%, 99% and 91% similarity with cation efflux family protein of *Pseudomonas stutzeri* DSM4166, copper resistance protein B of *Pseudomonas stutzeri* DSM10701 and copper oxidase gene of *Pseudomonas putida* H8234, respectively (Table 5). Inconsistencies in the gene sequences were observed. The amplified gene sequences showed similarity to *Pseudomonas stutzeri* DSM4166 and *Pseudomonas putida* H8234, but none of the isolates were identified as the above two. This could be due to the horizontal gene transfer from *Pseudomonas stutzeri* DSM4166 and *Pseudomonas putida* H8234 to the isolated strains.

Table 2: Comparison between the present heavy metal status in Bathinda with previous report by Hundal *et al*. (2006)

	Mean		
Metal	*Present study (mg kg^{-1})*	*Hundal et al. (2006) (mg kg^{-1})*	*Fold increase (approx.)*
Arsenic	0.3475	0.38	–
Boron	12.055*	1.02	12
Calcium	3162*	266.335	12
Cadmium	0.15*	0.03	5
Chromium	5.15*	0.05	103
Copper	2.84*	1.74	2
Iron	2823*	20.515	138
Potassium	526.6*	123.665	4
Magnesium	1098.12*	132.58	8
Manganese	45.265*	4.245	11
Phosphorus	120.362*	17.615	7
Sulfur	89.2825*	41.73	2
Zinc	24.53*	1.54	16

*Showed higher concentrations than Hundal *et al.* (2006)

Discussion

Present work focuses on an assessment of heavy metal content in the soil of Bathinda region, isolating the metal resistant *Pseudomonas* sp. and detecting genes responsible for multiple heavy metal resistance. Heavy metals are non-degradable and remain in the soil for a long period (Nies, 1999). Their contamination in agricultural soil and water bodies is of great concern as they can enter the food chain in a higher amount which eventually will affect the quality of food and safety (Matthews–Amune and Kakulu, 2012). Metal analysis of soil samples collected from three different sites showed that agricultural soil has more metal concentrations than the soil from the other two locations. Previous reports suggest that the agricultural soil irrigated with wastewater contains higher concentrations of heavy metals as compared to soils irrigated with normal water (Khan *et al*., 2008; Pathak *et al*., 2010). In India, previous studies suggest that fly ash from thermal power plants has a significantly higher concentration of heavy metal as compared to the garden soil (Ansari *et al*., 2011). According to Krishna and Govil (2007) soils in the vicinity of Surat industrial area which includes industries like NTPC, GAIL, HPCL, and ONGC have significantly higher concentrations of heavy metals as compared to standard values. In a recent study by Naseer and Pandey (2018) also showed that the soil around Bathinda is calcareous, alkaline and slightly saline. This explains the high calcium content in the soil in this study. Bathinda is an industrial, agriculture-dependent area with many industries such as National Fertiliser Ltd, Thermal Power Plant, Oil Refinery, Bathinda Chemical Factory. Effluents and ashes coming out of these industries and use of chemical fertilizers can be the reason behind the elevated level of metal content in the soil.

Metal contamination in the environment has limited the diversity of microbes. Only adapted microbes can survive, hence, the metal contaminated sites are the best possible source of metal resistant bacteria. The predominance of *Pseudomonas* implies that it is most resistant to heavy metal contamination. Many previous studies have shown *Pseudomonas* resistance towards high levels of heavy metal content, and they might have the potential of denitrification, nitrogen fixation, biosorption, degradation, accumulation, bioremediation and reduction of heavy metals and metalloids (Cánovas *et al*., 2003; Lalucat *et al*., 2006; Boricha and Fulekar, 2009).

Isolated *Pseudomonas* spp. has shown resistance not only to specific metal alone but also to combinations of Cu, Zn, and Fe. In many studies, bacterial isolates that displayed resistance to one metal also showed resistance towards several metals (Rajbanshi, 2009; Malik and Aleem, 2011). Isolated bacterial strains had shown their maximum resistance to Fe, and least to Zn. This indicates

Table 3: BLAST results of 16S rRNA gene sequencing analysis of eight bacterial isolates.

Strain	*Identification*	*Max. score*	*Total score*	*Query cover*	*E value*	*Identity*	*Accession number*
CCUPAS1	*Pseudomonas* spp. RA-4	2471	2471	100%	0.0	100%	KM594392
CCUPAS14	*Pseudomonas mendocina* strain MLCCp6	2510	2510	100%	0.0	100%	KM594393
CCUPAS16	*Pseudomonas mendocina* strain MLCCp6	2492	2492	100%	0.0	99%	KM594394
JCUPAS7	*Pseudomonas* spp. 418	2519	2519	100%	0.0	99%	KM594395
JCUPAS17	*Pseudomonas* spp. G1DM-23	2573	2573	100%	0.0	98%	KM594396
GCUPAS14	Gamma proteobacterium	1808	2579	100%	0.0	97%	Ambiguous sequence
GCUPAS16	*Pseudomonas citronellolis* strain TERIDB30	1884	1884	100%	0.0	92%	KM594397
GCUPAS20	*Pseudomonas* spp. RA-6	2392	2392	100%	0.0	99%	KM594398
CCUPAS6	*Pseudomonas mendocina* strain NaF-C-1	2507	2507	100%	0.0	100%	KM594399

Table 4: Highest MTC and MIC value recorded with corresponding strain

Metal		*Concentration (ppm)*	*Strain*
Cu	MTC	600	GCUPAS16
	MIC	700	
Zn	MTC	200	CCUPAS16
	MIC	300	
Fe	MTC	1500	CCUPAS1, CCUPAS6, CCUPAS14, CCUPAS16, JCUPAS17
	MIC	2000	
Cu+Zn	MTC	Cu50 + Zn100, Cu100 + Zn50	CCUPAS1, CCUPAS6, CCUPAS14, JCUPAS17, GCUPAS20
	MIC	Cu100 + Zn100	
Cu+Fe	MTC	Cu200 + Fe1500	CCUPAS14, GCUPAS14, GCUPAS20
	MIC	Cu300 + Fe1500	
Zn+Fe	MTC	Zn100 + Fe1000	All
	MIC	Zn100 + Fe1500	
Cu+Zn+Fe	MTC	Cu100 + Zn100 + Fe1000	GCUPAS20
	MIC	Cu100+Zn100+Fe1500	

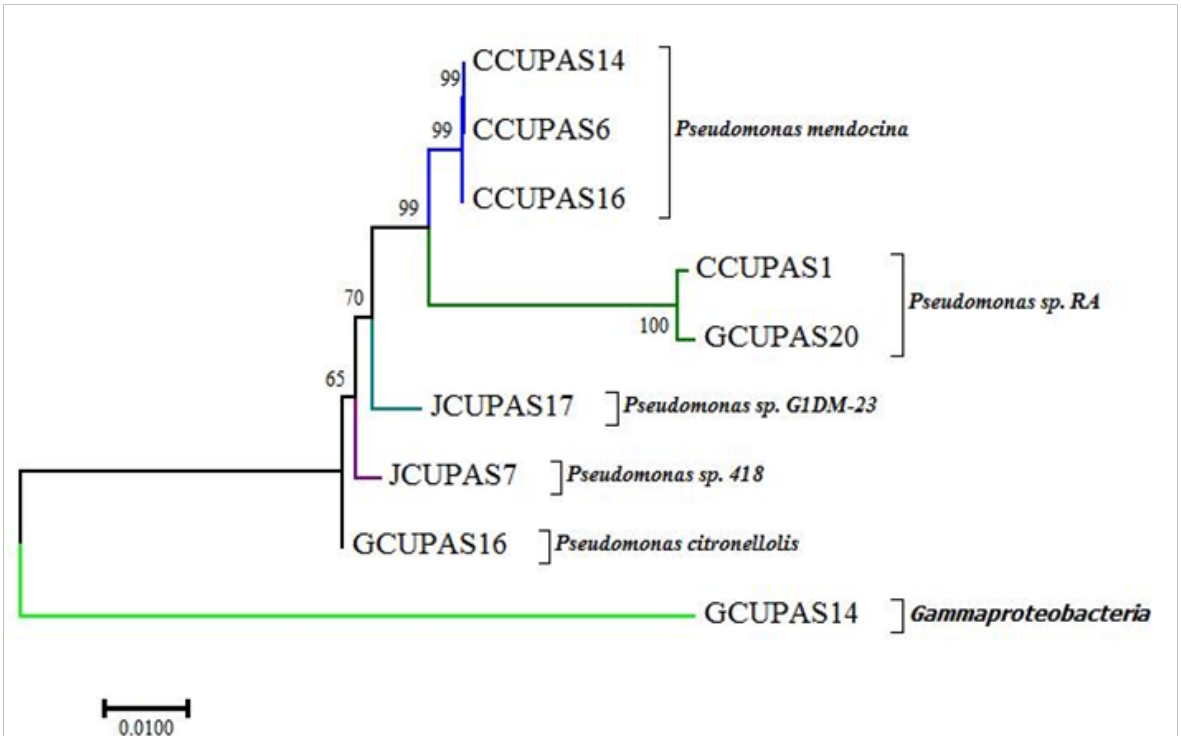

Fig. 1: Evolutionary relationship of nine bacterial isolates. Phylogenetic tree of 16S rRNA gene sequences of nine bacterial isolates was constructed inferred using the Neighbour-Joining method. Sequences are aligned by Clustal W. The optimal tree with the sum of branch length = 0.20683938 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method. Evolutionary analyses were conducted in MEGA 7 software

zinc is more toxic to isolated *Pseudomonas* spp. The resistance exhibited by bacterial isolates may be due to the presence of heavy metal resistance/reducing genes in their chromosomal DNA or extrachromosomal DNA (plasmids) (Silver and Phung, 1996). In this study, we tried to detect the heavy metal resistant genes present on the chromosomal DNA. We were able to detect three genes viz., *czcA, copB*, and *pcoA* by PCR amplification. czc operon encodes proteins that are involved in cation efflux system to detoxify the cell by actively extruding the heavy metal ions (Zn, Co, and Cd) out of the cell. CzcA encodes for CzcA protein which

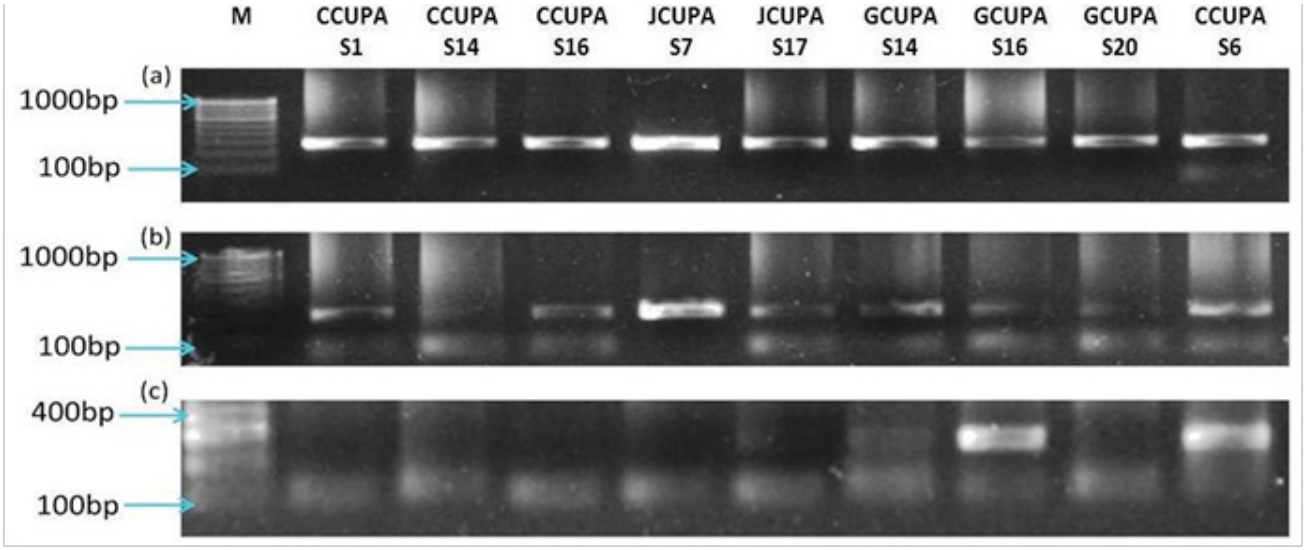

Fig. 2: 2% agarose gel showing amplified metal resistance gene *copB*, *czcA* and *pcoA*. L: DNA ladder, a) *copB*, b) *czcA*, c) *pcoA*

Table 5: BLAST results of metal resistance gene sequences.

Gene	*Description*	*Max score*	*Total score*	*Query cover*	*E value*	*Identity*
copB	*Pseudomonas stutzeri* DSM 10701, complete genome. Feature: copper resistance protein B	388	388	45%	2e-104	99%
czcA	*Pseudomonas stutzeri* DSM 4166, complete genome. Feature: cation efflux family	294	294	100%	1e-76	100%
pcoA	*Pseudomonas putida* H8234, complete genome. Feature: copper oxidase	878	878	100%	0.0	91%

is an root nodulation development (RND) protein. It provides resistance to bacteria in combination with CzcB and CzcC proteins. *czcA* gene was previously reported in highly zinc resistant bacteria *Comamonas testosterone* S44. Also, the expression of *czcD* and *nccA* was increased with Co and Cd exposure in *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Pseudomonas* spp. and *Bordetella* spp. (Abdelatey *et al.*, 2011). cop operon and pco operon are responsible for copper homeostasis. Previously *cop* genes are detected in the chromosome of *Pseudomonas* spp. (Mellano and Cooksey, 1988). Palmieri *et al.* (2010) demonstrated that under copper stress, the expression of *copA* and *copB* in *Xanthomonas axonopodis* is upregulated. In this study, the presence of *copB* in all the nine isolates could be responsible for tolerance showed by nine isolates against Cu. pcoABCD is another operon involved in maintaining copper homeostasis and it was first detected in *E. coli*. PcoA is involved in protecting periplasmic enzymes from damage induced by copper. Absence of PcoA has induced hypersensitivity in cells towards copper (Lee *et al.*, 2002). In this study, *pcoA* gene was detected in two isolated bacterial strains i.e., GCUPAS16 and CCUPAS6. GCUPAS16 has shown maximum tolerance to copper and the presence of both *cop* and *pco* genes could be responsible for maximum tolerance.

The present study suggests that the predominant bacterial species isolated from metal contaminated soil is *Pseudomonas* and have multiple metal tolerance. Many previous reports suggest that *Pseudomonas* sp. possesses the potential of denitrification, nitrogen fixation, biosorption, degradation, accumulation, bioremediation, and reduction of metals. Hence, metal resistant *Pseudomonas* sp. can be further exploited for the bioremediation purpose and /or Potential plant growth promoting bacteria.

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AA has mainly executed the work; RS has assisted in gene sequencing and MEGA analysis. PB has done the sequencing of the 16S rRNA gene. SK has conceptualized the work and prepared the final manuscript. Authors are thankful to the Central University of Punjab, Bathinda for providing the necessary infrastructure.

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