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Impact of *Vetiveria zizanioides* rhizosphere bacterial isolates on PGPR traits and cadmium resistance

*W. Jabez Osborne, V.S. Saravanan, Amitava Mukherjee, N. Chandrasekaran**
Nanobiomedicne Laboratory, School of Biosciences and Technology, VIT University, Vellore, Tamilnadu, India
**Corresponding Author, Email: nchandrasekaran@vit.ac.in*

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Abstract

In our study we have proved that most vetiver rhizosphere bacterial isolates are potent biomolecule synthesizers. We isolated culturable bacterium associated with vetiver rhizosphere using three carbon sources and phenotypic characterizations were performed. The medium and cultural conditions of the isolates were optimized under shake flask conditions. Further, the isolates were assessed for their ability to synthesize biomolecules and plant growth promoting rhizobacterial (PGPR) traits. Siderophore production was determined in all the tested isolates; Phosphate solubilization was performed and seven isolates were shown to solubilize phosphate. Indole 3-acetic acid (IAA) was produced by all isolates when grown on MS medium supplemented with tryptophan and the amount of IAA produced were quantified with standards. Metal tolerance concentration (MTC) was performed and it was observed that most isolates were able to survive till 300mg L^{-1} on cadmium amended minimal medium. The isolate (VITJCSKK14) was able to show resistance till 500mg L^{-1}. Solubilization of zinc metal (0.1%) was analysed using LGI medium; halos were observed and quantified by checking the growth, pH and optical density. The soluble Zn present in the culture broth was determined using an atomic absorption spectrophotometer (AAS-Model Varian C) at different periods of growth and the maximum solubilization was recorded after 120 h with a 0.1% Zn metal amendment was 634mgl^{-1}. Further the intracellular proteins were separated to observe the whole cell protein and stress tolerant proteins from VITJCSKK14. The intracellular proteins were extracted from the cultures grown in Tris minimal media supplemented with cadmium and quantification was performed using Bradford's method. The proteins were separated on SDS-PAGE and the stress protein bands were observed. It was found that high molecular weight protein was appeared in the test sample. The molecular taxonomy of the active isolate VITJCSKK14 was carried out by 16S rRNA analysis and phylogenetic tree was constructed using CLUSTALV software. Based on the phenotypic and phylogenetic analysis, the isolate VITJCSKK14 was identified as *Acinetobacter* Sp. This study also gives a hypothesis that *Vetiveria zizanioides* rhizosphere bacterium may aid in plant growth promotion and their survivability in adverse conditions.

1. Introduction

Vetiver grass is a perennial grass of Gramineae, which is originated from India and Africa continent (Xia et al., 1998) and is distributed worldwide. Vetiver often regarded as miracle plant can survive in infertile lateritic soil or flooding, they also prevent soil erosion and maintains soil moisture content (Leaungvutiviroj et al., 2006). Certain germlines of the species *Vetiveria zizanioides* have long been cultivated for their odorous roots that contain the essential oil of Vetiver, used extensively in perfumery and cosmetics (Maffei, 2002). Vetiver grass has a wide root system consisting of long, fibrous roots and rootlets forming a sort of fasciculate mass, extending 2-3 m deep hence aids soil and water conservation. Root tissues contain oil-producing cells, responsible for its characteristic odor (Peyron, 1989). Vetiver oil is one of the most complex mixtures of sesquiterpene alcohols and hydrocarbons, and also one of the most viscous oils with an extremely slow rate of volatility. It is used extensively for blending in cosmetics, in the soap industry as a odor contributor in bases such as chypre (Weyerstahl et al., 1996) and rose (Chowdhury et al., 2002), in several masculine fragrances, and as a fixative in the perfumery industry prolonging the life of any composition to which it is added (Lemberg and Hale, 1978; Akhila and Rani, 2002).. Luigi Del Giudice and his colleagues were the first to study the root-associated bacteria and showed that most of them are able to grow by using oil sesquiterpenes as a carbon source (Guidice et al., 2008) It is also told

that vetiver is capable of tolerating high concentrations of various heavy metals without any effect on its growth and development (Andra et al., 2009). Zinc is considered as a double edged sword in living systems since at trace concentrations, it acts as a cofactor in enzymes belonging to all six classes recognized by IUBMB nomenclature (Saravanan et al., 2007).

Cadmium and zinc are common heavy metal pollutants in mine lands and they often occur together in the environment because of their similar chemical properties(Hutton, 1983).Cadmium is found to disturb ionregulatory system and oxidative stress wheras zinc aids as a cofactor for many enzymes at low concentration. In high level zinc may cause osmoregulatory disturbances.

Plant growth promoting rhizobacteria (PGPR) are root colonizing bacteria with beneficial effects including plant growth promotion and disease control. PGPR strains are reported to produce a variety of metabolites which play an important role in induced systemic resistance (ISR) against many plant diseases (Mavrodi et al., 2001). The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Arshad and Frankenberger, 1993; Glick, 1995), (ii) asymbiotic N2 fixation (Boddey and Dobereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan et al., 1992) and cyanide (Flaishman et al., 1996), (iv) solubilization of mineral phosphates and other nutrients (De Freitas et al., 1997; Gaur, 1990).

In this study we have isolated bacterial community from rhizosphere of vetiver by culture dependent technique. The isolates were screened for its plant growth promoting activity which might help in continued existence of plant in unfavourable conditions

2. Materials and Methods

1.1 Plant sampling and location:

Vetiver (*Vetiveria zizanioides*) plant was collected from hills of Kannamangalam, Kerala, India. Three plant samples were collected from different location within 30m carefully the soil sample was kept intact with rhizosphere and soil samples were processed within 2 h of collection.

1.2 Culture medium used for isolation:

The strains were isolated by inoculating serially diluted samples onto N-free LGI medium (Cavalcante and Dobereiner, 1988) was used for the isolation of N_2 fixers, PCAT and Trypticase soy agar. All the plates were incubated for 3-4 days at 30°C and isolates were purified based on different colony morphology.

1.3 Phenotypic characterization:

Isolates were grown at solid medium to determine its phenotypic characteristic, further its ability to grow in various carbon sources were analysed using LGI medium and tests such as oxidase, catalase and H_2S production were also performed.

1.4 Cadmium metal toxicity testing:

Resistance to heavy metals was determined by an agar dilution method (Washington and Sutter 1980). Plates containing 20ml of agar and different concentrations of metal were poured on the day of the experiments. The concentrations for Cadmium metal tested were as follows (in mg L^{-1}): 100, 200, 300, 400 and 500. Plates were dried at 37°C for 30 min and inoculated with 0.1 ml from exponentially growing cultures. The plates were incubated at 30°C for 2 days. Plates containing media with no added metal were inoculated in the same way to act as controls. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of metal ion preventing growth. For the purpose of defining metal resistance, those strains which were not inhibited by 500 mg L^{-1} Cd were regarded as being resistant (Sabry et al., 1997).

1.5 SDS-PAGE of metal tolerant proteins:

SDS PAGE was performed with and without broth supplemented with metals and the bands were compared.

1.6 Detection of siderophore:

Siderophores were detected using the universal chemical assays on modified chrome azurol-S agar plates as described previously (Schwyn & Neilands, 1987) Aliquots of cultures were inoculated and incubated for 72 h at 30°C. Orange haloes that formed around the colonies on blue agar were considered indicative of siderophore production. The concentration of siderophore was quantified.

1.7 Production of Indole acetic acid:

Qualitative analysis of Indole acetic acid was assayed in UV-Spectrophotometer at 530nm (Gordon and Weber, 1951).

1.8 Phosphate Solubilization:

Isolates were tested using Pikovskaya medium agar plates containing insoluble tricalcium phosphate as the sole phosphorous source. The solubilization haloes were observed after incubation at 30ºC.

1.9 Zinc Metal solubilization:

Solubilization of metal was determined with the strains isolated from vetiver rhizosphere soil were tested on Zn. The solubilization potential was assessed both qualitatively and quantitatively under *in vitro* conditions.

Qualitative analysis

The isolates were inoculated into LGI medium amended with zinc metal powder, incubated at 30ºC for 48 hours. The diameters of the clearing zones around the colonies were measured.

Quantitative analysis

The bacterial isolates were inoculated separately onto 100 ml, LGI medium supplemented with Zinc metal powder (0.1 %) in 250 ml Erlenmeyer flasks. Then the flasks were inoculated with 2 ml of the test culture with a cell load of 10^7cells ml^{-1}. Three flasks were maintained with an uninoculated control for each treatment. The samples were withdrawn at 24, 48, 72, 96 and 120 h intervals, centrifuged to remove the debris and cells. The supernatant of the broth was used for determination of available metal content using Atomic Absorption Spectrophotometry (AAS) (Saravanan et al., 2007).

1.10 Identification of bacteria by 16S rRNA technique:

Bacterial strains were characterised using the primers27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3') DNA was extracted from cells and the 16S rRNA sequence was determined by the fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on a ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic Analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalV software, and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix (Poonguzhali et al., 2008).

3. Results

3.1 Isolation and characterization

Bacterial community in Vetiver were isolated from Kerala using PCAT medium, N-Free LGI medium and Trypticase Soy agar. A total of 26 isolates were cultured, purified in their corresponding medium and was maintained in nutrient agar medium for further screening. The biochemical characterization showed that most isolates belonged to gram negative bacteria. (Table 1).

3.2 Cadmium metal toxicity testing

The percentages of the isolates resistant to various concentrations of Cadmium heavy metal ions were determined and 77 % of the isolates were resistant to Cd upto 300 mg L^{-1}**.** Strain VITJCSKK14 was resistant to a concentration of about 500 mg $L^{-1}$and it was used for further studies (Fig 1 & 2).

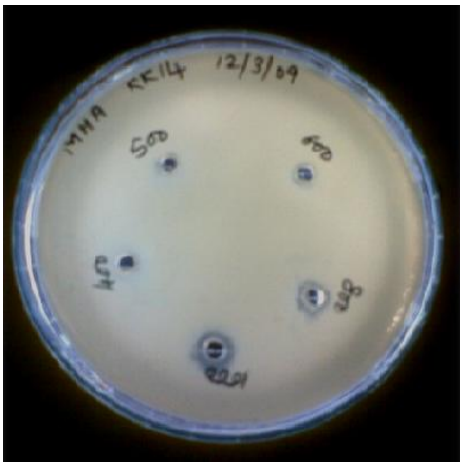


Fig. 1. Representative well diffusion assay plate containing minimal medium dosed with 400, 500, 600, 800, 1000 ppm Cadmium chloride, and inoculated with 10^3 to 10^4 cells of *Acinetobacter Sp.*VITJCSKK14, after 24 h incubation at 30°C.

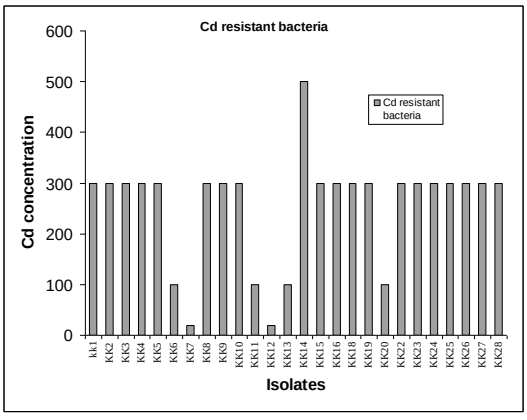


Fig. 2. Graph showing cadmium tolerance in various concentrations.

Table: 1 Biochemical Characteristics of the isolates

ISOLATES	BIOCHEMICAL CHARACTERISTICS									CARBON UTILIZATION							
	Gram's	Endo spore	Indole	MR	VP	Citrate	Catalase	Oxidase	TSI	A	D	F	G	L	M	R	S
KK1	+ rods	+	+	+	-	+	+	-	k/a-	-	+	-	+	-	-	-	-
KK2	-	-	-	-	+	+	+	+	k/k-	-	+	-	+	-	-	-	-
KK3	-	-	-	-	-	+	+	+	k/a-	+	+	-	+	+	+	-	+
KK4	-	-	-	-	-	+	-	+	k/a-	-	+	-	+	+	-	-	V
KK5	-	-	-	-	-	-	-	-	k/a-	-	+	-	+	-	-	-	-
KK6	-	-	-	-	-	+	+	+	k/a-	V	+	-	+	+	V	-	+
KK7	+	+	-	-	-	+	-	+	k/a-	V	+	-	+	-	V	-	+
KK8	+	+	-	-	-	+	+	+	a/a +	-	+	-	+	-	-	V	V
KK9	-	-	+	+	-	-	-	+	k/k-	+	+	+	+	+	+	+	+
KK10	-	-	-	-	-	+	+	+	a/a+	-	+	-	-	-	V	-	-
KK11	-	-	+	+	-	+	+	+	k/a-	+	+	+	+	+	+	+	+
KK12	-	-	-	-	+	+	+	+	k/k-	+	+	+	+	+	+	+	+
KK13	-	-	+	+	+	+	+	+	k/k-	+	+	+	+	+	+	+	+
KK14	-	-	-	-	-	+	+	-	k/a-	+	+	+	+	+	+	+	+
KK15	-	-	-	-	+	+	+	+	k/a-	+	+	+	+	+	+	+	+
KK16	+	+	-	-	-	+	+	+	k/a+	+	+	+	+	+	+	+	+
KK18	-	-	-	-	-	+	+	+	k/a-	+	+	+	+	+	+	+	+
KK19	-	-	-	-	-	+	+	+	k/k-	-	+	+	+	-	-	-	+
KK20	-	-	+	+	+	+	+	+	k/aH_2S	+	+	+	+	+	+	+	+
KK22	-	-	-	-	-	+	+	+	a/aH_2S	-	+	-	-	-	+	-	-
KK23	-	-	-	-	-	-	+	+	k/a-	+	+	-	-	+	+	-	-
KK24	-	-	-	-	-	+	+	+	k/k-	+	+	+	V	+	+	-	+
KK25	-	-	-	-	-	+	+	+	k/k-	-	+	+	-	-	-	-	-
KK26	+	+	-	-	-	+	+	-	k/a-	+	+	-	-	+	+	-	+
KK27	-	-	-	-	-	+	+	-	k/k-	+	+	-	-	+	+	-	-
KK28	-	-	-	-	-	+	+	-	k/a-	-	+	-	-	+	-	-	-

+ = Positive;- = Negative; V =Variable; TSI – Triple Sugar Iron, A – Arabinose, D- Dextrose, F- Fructose, G – Galactose, L- Lactose, M – Maltose, R – Rafinose, S - Sucrose; a / a = Glucose and sucrose and/or lactose fermented; a / a+ = Glucose fermented with gas production, sucrose and/or lactose fermented; k / a = Glucose only fermented; k / a+ = Glucose only fermented with gas produced; k / k = No sugars fermented; k / H_2S = Lactose and sucrose not fermented, H_2S production (black butt) is all that can be seen; a / H_2S = Lactose and/or sucrose fermented, H_2S production (black butt).

3.3 Plant Growth promoting traits

3.3.1 Siderophore detection

In this work the production of siderophores was successfully carried out, the detection of siderophores was made on agar and liquid media. It was found that about 40 % of the isolates produced siderophore and strain VITJCSKK14 produced increased levels of siderophore (Table: 2).

3.3.2 Indole acetic acid synthesis

All the isolates were capable of producing indole acetic acid in various levels, Quantitative assay of all the selected strains of vetiver were capable of producing considerable amounts of IAA. Almost 75 % of the strains produced more than 50 mg L^{-1} (Table: 2).

3.3.3 Phosphate Solubilization

A total of 7 isolates were able to solubilize the insoluble tri calcium phosphate supplemented in the Pikovskaya agar plates after incubation when the cultures were spotted at the centre of the plates(Table: 2).

3.3.4 Zinc Metal solubilization

Solubilization of Zn metal was performed in solid plate and halo's were observed after 48 hours. The solubilization ability was also assessed in broth assay and was compared with our previous study with *Gluconacetobacter diazotrophicus* (Saravanan et al., 2007), wherein the halos were observed in 24h in this study the halos were formed in 36h. Subsequently within 24h pH of the broth reduced to less than 4 and after 120 h the zinc available in the broth was found to be 634 mg L^{-1}. The data is described in figure 3.

Table: 2. PGPR Activities of Rhizobacteria

Strain no	Siderophore production		Phosphate Solubilization	IAA production
	Siderophore Production	Quantitative analysis (µg/ml)		Quantitative analysis (µg/ml)
KK1	-		V	24± 1.15
KK2	+	43.66± 0.33	+	73.6± 1.8
KK3	-	-	-	62.3±0.88
KK4	-	-	+	58.6± 0.33
KK5	-	-	V	34.6± 1.20
KK6	-	-	+	43.6± 0.88
KK7	-	-	-	82± 1.15
KK8	-	-	-	186.66± 1.20
KK9	-	-	-	56± 1.73
KK10	-	-	-	62.66± 1.20
KK11	+	65.66± 0.33	V	105.3± 2.40
KK12	+	80.3± 1.20	-	110.3± 2.60
KK13	+	34.66± 0.88	V	111.66± 1.45
KK14	+	80.33± 0.86	-	63.33± 1.45
KK15	+	44.66± 0.33	+	155± 1.73
KK16	+	53.66± 0.88	+	161± 1.0
KK18	+	55.66± 0.88	+	154± 2
KK19	+	62.1± 0.32	-	25.33± 0.66
KK20	+	71.3± 0.92	V	103± 0.57
KK22	-	-	-	66± 1.15
KK23	-	-	-	136.33± 0.88
KK24	-	-	-	45± 0.57
KK25	-	-	-	43.6± 1.45
KK26	-	-	-	61.33± 1.45
KK27	-	-	+	18.6± 0.88
KK28	-	-	-	124± 1.73

Values are mean ±SE of three replicates

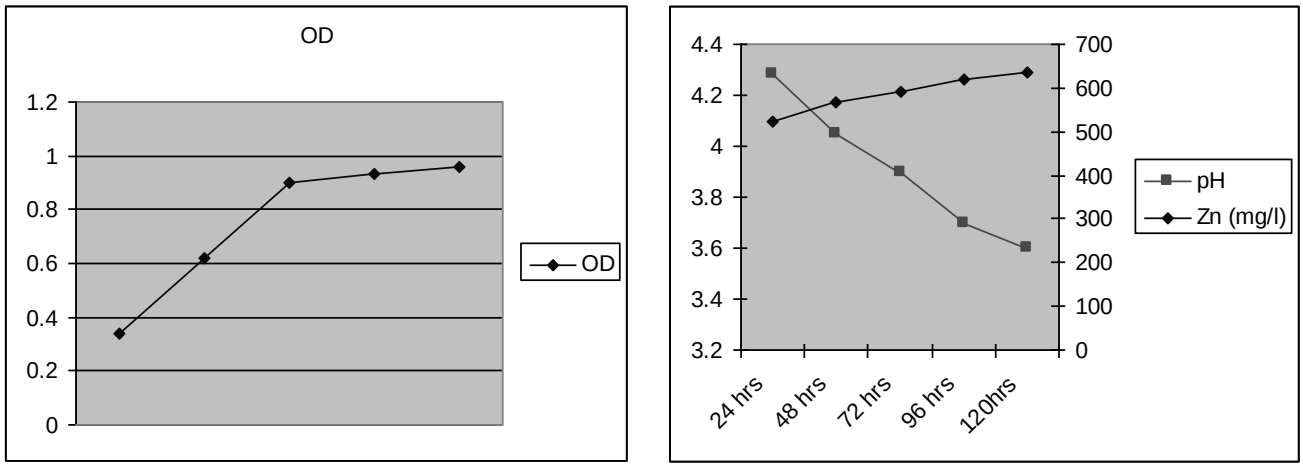


Fig 3 **A**. Changes in population of *Acinetobacter Sp* VITJCSKK14 culture grown in Zn metal (0.1%)-amended LGI medium. **B**. Changes in pH and available Zn in LGI medium amended with Zn metal during growth of *Acinetobacter Sp* VITJCSKK14.

3.4 Stress protein using PAGE

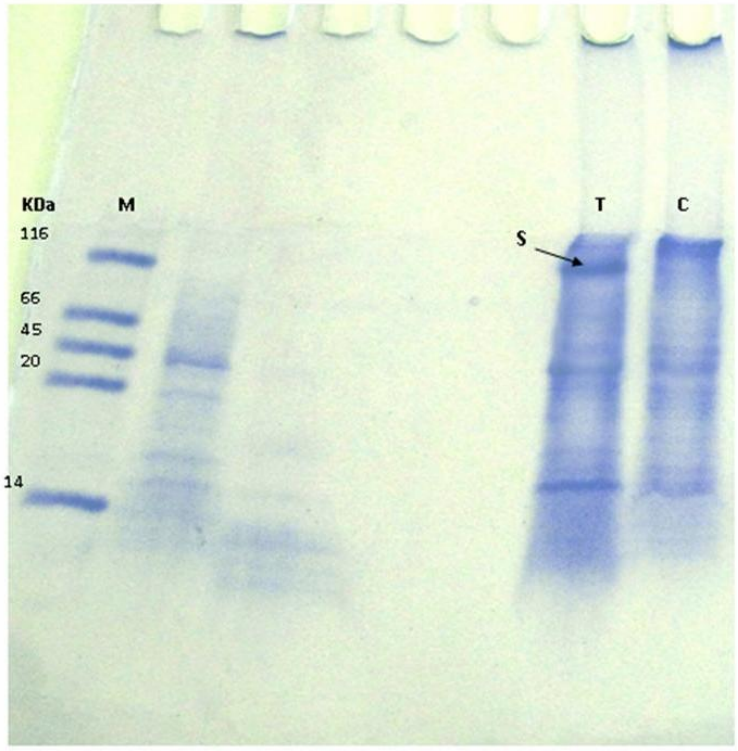
Protein separation using SDS PAGE showed that a stress protein band with a high molecular weight was obtained. (Fig 4)

3.5 Taxonomical identification

The identification by 16S rRNA gene sequencing analysis ascertained the bacteria belong to gamma proteobacteria and it had a closed

relationship with Acinetobacter sp. The gene sequence is been submitted to the GenBank under the accession no GQ200824. A phenogram reflecting the relationship among the strains and candidate sequences of related strains obtained from the NCBI database is presented in Fig 5.

Fig 4 SDS PAGE showing stress protein



M-Marker, T-Test, C-Control, S – Stress Protein

4. Discussion

It is known that rhizosphere region of plants remains as the major source for microbial population because of its rich nutrient availability (Jagnow, 1987). Recent studies by Del Guidice et al. (2008) reveal the various bacterial communities of Vetiver plant. In our study we have isolated and characterized efficient bacterial isolates from the vetiver grass resistant to cadmium. We reported a strain similar to *Acinetobacter* Sp from the rhizosphere soil of vetiver.

Further this isolate was capable of surviving upto 500 mg L^{-1} Cadmium and previously it is reported that vetiver can uptake Zn, As, Cr, Pb, Se and cadmium upto a concentration of 20 mg L^{-1} (Truong et al., 2003). So these bacterial isolates of vetiver may play a major role in providing resistance to the plant. There are mechanisms for bacterial resistance towards toxic metals and studies also reveal that reduction of heavy metals increases the activity of plant growth (Burd et al., 1998). The analysis of solubilization of zinc metal shows that the presently isolated strain solubilized Zn metal equal to that of *Gluconacetobacter diazotrophicus*. There was a decrease in the pH throughout the experiment as acid is produced in bacterial metabolism. An increase in the concentration of Zinc was observed which might possess toxicity to the culture. The strain VITJCSKK14 was capable of surviving at high concentrations of Cadmium and Zinc.

The isolate tested was found to possess good activity for various plant growth promoting traits, it was an effective synthesizer of certain molecules like indole acetic acid, Siderophore and phosphate solubilization. However the amount of IAA produced is directly proportional to the amount of precursor present in the plant (Arshad and Frankenberger, 1993). It is evident that increased uptake of nutrients and phytohormones production by microorganisms may play a role in plant growth promotion (Ryu et al., 2006). The strain isolated produced siderophore comparatively higher than the other isolates. Siderophores chelates iron and other metals contributing to suppression of disease by conferring a competitive advantage to biocontrol agents (Hofte et al., 1992). Phosphate solubilization was observed in few isolates, as there is acid

production during bacterial metabolism which in turn might aid in the solubilzation of phosphate.

The protein content present in the metal treated culture broth was compared with the untreated culture broth (Whole cell protein content). A band was obtained in a range of about 116 Kda which was not seen in the untreated broth, illustrates that the protein band belongs to a high molecular weight protein. This protein is referred as stress protein since it is expressed only because of the cadmium stress provided in the broth.

Vetiver is a fast growing grass that can grow upto 1m in a month. The rhizosphere bacterial community may play a pivotal role in this, as they are efficient in nutrient uptake and synthesis of phytohormones. The rhizobacterial load may also aid in the increased uptake of metals from the rhizosphere into the vetiver plan. This study describes that the VITJCSKK14 is a capable PGPR and heavy metal resistant bacterial strain. Presently supplementation of efficient strains in *invivo* conditions for the enhanced uptake of metal and growth is under progress.

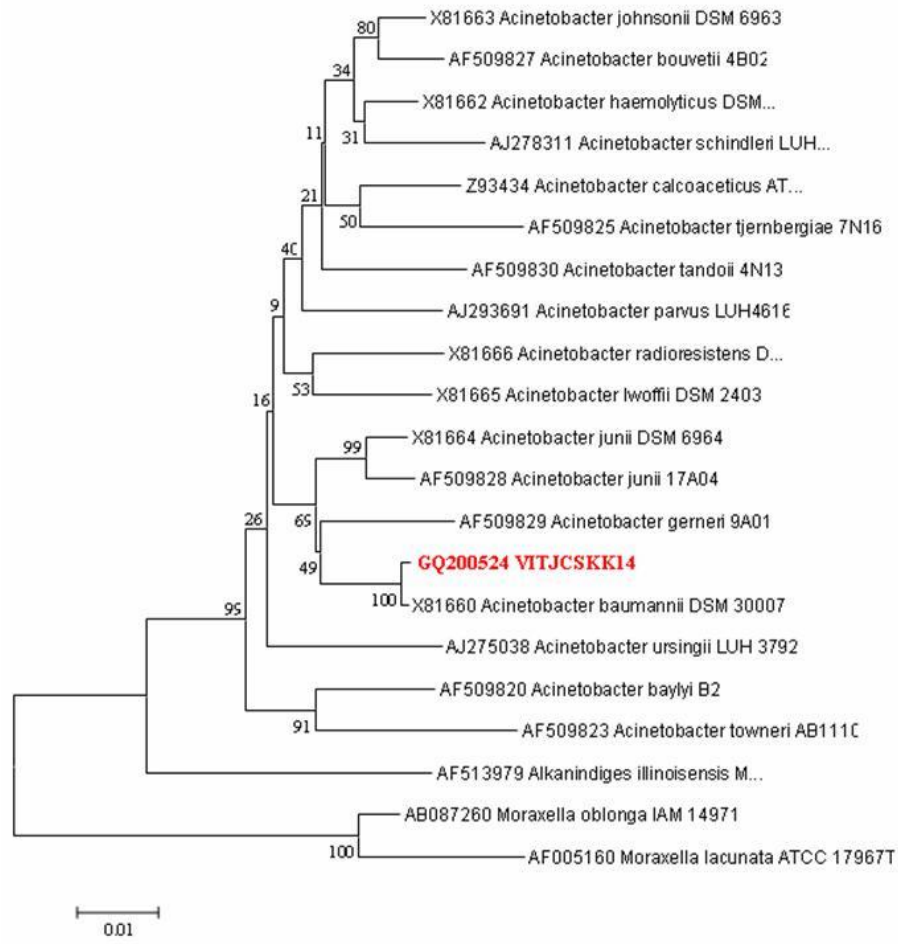


Fig 5 Phylogenetic relationships of the isolate from vetiver based on 16S rRNA sequence and related sequences. The tree was constructed using closely related sequences based on Euclidean distance (neighbour joining algorithm with kimura parameter 2). The numbers of the nodes indicates the levels of bootstrap. Sequence accession numbers are indicated before the genus name.

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