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Copper resistant bacteria at coal mines sorrange Balochistan

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Abstract

Current study was carried out to isolate and characterize copper (Cu) resistant bacteria dwelling in the coal mines of Sorrange, Balochistan, Pakistan. Sanger sequencing of 16S rRNA gene has performed to decipher characterization of bacteria. Initially resistance to varying concentrations (400µg/ml-750µg/ml) of Copper (Cu) was developed on 13 isolated strains and after that morphological, biochemical and molecular analysis was performed. The genomic DNA was extracted. PCR reactions were performed using DNA primers specific for 16S rRNA gene "27f and RD1". Sequence analysis of 16S rRNA gene resulted in 96-100% identical microorganisms to the following bacteria; *Bacillus thuringiensis serovarkonkukian* str., *Bacillus weihenstephanensis* KBAB4., *Micrococcus luteus* NCTC 2665, *Bacillus anthracis* str. *Sterne, Bacillus cereus* ATCC , *Arthrobacterarilaitensis*RE117, *Bacillus anthracis* str. *Ames, Bacillus pseudomycoides* DSM12442, *Bacillus toyonensis* BCT-7112and *Bacillus subtilis* subsp. *subtilis str*. We have constructed the Phylogenetic linkage tree for all these bacteria and results indicate that quite a few of our isolates may have a potential use for biodegradation of heavy metals from diverse environments.

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Introduction

Metals are the part of earth crust since the evolution, even when the initial life on the earth came in to being was in the presence of plenty of heavy metals. The living organisms use some metals as vital component of food but with the advancement in studies it is identified that only a few are toxic for living creatures and to the environment (Choudhury and Srivastava 2001).

Environmental pollution is increasing day by day world widely because of innovation and expansion of industrialization progress, urbanization, anthropogenic activities and the increasing population level. (Khan 2000) along with the other heavy metal contaminants by industries operations including mining, smelting, metal forging, preparation of alkaline storage batteries and combustion process (Kumar *et al.,* 2011) are also adding to the water (Rajaganapathy *et al.,* 2011) and soil (Benson 2006). Furthermore metal can be added to soil through agricultural waste containing insecticides and pesticides, and addition of sewage sludge (Rajaganapathy *et al.,* 2011).

With evolution Microbes have developed certain mechanisms, enabling them to biochemically degrade the toxicity of heavy metals, making them tolerant to that specific metals. The most important mechanisms for adoption of their resistance are; an active metal efflux, synthesis of metal-binding peptides, proteins or polysaccharides such as metalothioneins, extra cellular polymeric substance (EPS) and the increasing the expression of detoxification enzymes(Gadd 2004).

Copper (Cu) is a key metal which is toxic at far above the ground concentrations. In soil cu is supplemented by mining, smelting, Industrial process like wood impregnation and by the agricultural waste, including insecticides and pesticides (Komárek *et al.,* 2010) and modification with the manicure having copper (Bolan *et al.,* 2004). Copper is abundantly present in the environment, likewise in the earth crust.

It is a vital factor of a living body taking part in a number of life processes. Copper may combine to the soil particles like organic matter, clay mineral, Al, Fe and Monoxides reaching to a high concentration in the soil and soil surface.

In soil copper is involved in numerous of process including; biochemical cycling of the elements, plant growth, decomposition of organic matter, maintenance of soil structure, detoxification and pest control (Giller *et al.,* 1998). However, Cu deposition in the soil becomes adverse for the soil bacteria that in turn impact the biological processes. as the soil bacteria involve in these processes as well as the soil quality (Altimira *et al.,* 2012, Dell'Amico *et al.,* 2008).

Traditional techniques used for bacterial identification but these have limitations. As the technology of PCR and DNA sequencing is improving, aligning of gene sequences of bacterial species demonstrated that the 16S rRNA gene is highly conserved within a species and species of same genus. So, it can be used as a new tool for recognition of the bacteria to the species level. Following this new standard, phylogenitics trees based on base differences between species are developed and bacteria are classified and reclassified into new genera heavy metal-resistant bacteria strain was isolated from heavy metal-contaminated soils and identified based on the 16S rDNA (Jiang *et al.,* 2008). In the light of above facts the present study was aim to isolate and characterize at molecular level the bacteria show resistant to Copper and will be studied its different growth characteristics like morphological, biochemical, under various conditions and metal stress, so as to ensure that the isolate could be efficiently employed for bioremediation of toxic metal from the environment.

Materials and methods

Soil samples were collected (0-15cm depth) from different sites in the coal mines of Sorrange Balochistan using aseptic conditions in sterilized jars and were kept at 4°C. Metal salts were used to prepare stock solutions of concentrations 1gm $CuSo_4$. $5H_2O$/100ml autoclaved distilled water (Narasimhulu *et al.,* 1948, Singh *et al.,* 2010).

Isolation of bacteria to get pure culture

Nutrient agar and brain heart infusion mediums were used to isolate the bacterial strains from the samples**.** The 10-^{6} dilution of sample was spreaded to the nutrient agar plate and incubated for 24-48 hours at 30-37^{o}C. Streaking method was used to separate bacteria from dense population and incubated for 24-48 hours at 30-37^{o}C.To grow resistant bacterial strains a specific media with heavy metals was prepared, the above prepared stock solutions were added to the nutrient media in respective concentrations just after the autoclaving, ranging from 50µg/ml to800µg/ml.

Concentrations were prepared by using the following formula;

$C_1V_1=C_2V_2$.

Where;

C_1=Concentration of metal in agar & C_2=Metal Concentration in stock solution.

V_1=Volume of agar & V2=Volume of stock solution used.

$V_2= C_1V_1/C_2$

The above pure cultures were applied to them by streaking and incubated at 30-37^{o}C for 24 to 48 hrs. The effect of varying concentrations of metals on bacteria tolerance was examined for 24 hours at 37^{o}C (Singh *et al.,* 2010).

Determination of MIC (minimum inhibitory concentration)

These inoculated cultures were grown on ascending concentrations of copper, until no growth is observed (50µg/ml-800µg/ml). The lowest concentration at which the organisms stop to grow more is MIC and values were noted (Singh *et al.,* 2010).

Identification and Characterization of Microorganisms

Morphological characterization

Gram staining is a differential staining method, was used to distinguish bacteria on the biases of chemical composition of cell wall. Spore staining test was performed to characterize bacteria according to their ability of producing spores.

Biochemical tests

IMViC (Indole- Methyl Red- Voges Proskauer and Citrate utilization test)

Indole test

Indole production is detected by Kovac's or Ehrlich's reagent. Tryptone broth was prepared, autoclaved and poured 6ml in each test tube. Sample was inoculated from the primary plate. incubated at 37^{o}C for 18-24hrs. After incubation 0.3ml of Kovak's reagent was added to culture fluids.

*MRVP test***.**

An MRVP broth was prepared, autoclaved and divided equally for both methyl red test and Voges Proskauer test. 5ml of MRVP broth was poured into test tubes and were inoculated by 24 hours fresh cultures and left for 24 hours in incubation at 37^{o}C.Methyl red indicator was added and color change was observed. Voges Proskauer test was used to detect acetone in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the VP broth.

Citrate

The green colored Simmon's citrate agar Slants were prepared and inoculated with fresh culture on entire surface of slants, incubated at 35^{o}C for 24-48hrs.The alkaline pH turns the pH indicator (bromthymol blue) from green to blue.

Catalase

Catalase test was performed to identification of catalase producing microorganisms. Smears of fresh cultures were prepared in water droplet on glass slide using sterilized loop. The smear was air dried and then single drop of hydrogen per oxide were applied on smear. Frequent bobble appearance were observed on subjected area.

SIM

SIM is differential medium that tests three different parameters, which are abbreviated by the three letters in the name sulpher, indole and motility. The bacterial inoculation was done deep in the semisolid media and was incubated at 37^{o}C for 24 hours.

Motility test was performed by assessing the turbidity of medium. The turbidity along the straight line of inoculums indicates the presence of motile bacteria.

Molecular assessment

DNA extraction protocol

CTAB based protocol way used for the extraction of bacterial genomic DNA.CTAB buffer was heated at 65°C, for about 10-30 min in water bath. Then 10-20mg bacterial cells were taken, these cells were obtained by centrifuging the 24 hours old broth cultures with distilled water at 12,000rpm for 10min. The supernatant was discarded; pallet consists of the bacterial cells. 700µl of preheated CTAB buffer was addedin the tubes containing the bacterial cells and 20-30 µl of β-Mercaptoethanol was added to stop oxidation, after that tubeswere mixed vigorously and incubated at 65°C for 20min.Followed by 300µl ammonium acetate (3Mol, pH: 5.2). Was added, kept on ice for 20min and centrifuged at 12,000rpm for 10min. The supernatant was shifted in new tubes and the sediment was discarded. Now equal to the volume of the supernatant 24:1 (chloroform: isoamylalcohol) was added to remove the protein and centrifuged them for 10min at 12,000rpm (4°C). The step was repeated.

The tubes were then incubated at -65°C (ultra-freezing) for 30min to sediment DNA, centrifuged at 12,000rpm for 10min (4°C)and the supernatant was drained out. The pallet was then washed with 75% ethanol, twice and eluted in 50µl of DD water or 1X TE buffer and stored at -20°C (Cheng *et al*., 2003). Verification of genomic DNA was done by gel electrophoresis.

PCR (polymerase chain reaction)

Universal primers for 16s r RNA were used to amplify bacterial DNA of the isolated samples.

Table 1. List of primers used in the experiment.

Primer name		Sequence	Base pairs	Product size
27f	Forward primer	AGAGTTTGATCCTGGCTCAG	20	
RD1	Reverse primer	AAGGAGGTGATCCAGCC	17	1500bp

Sequencing and BLAST

Sequencing was done commercially (MACROGEN laboratory, South Korea). BLAST was performed for finding similarities with other sequences. Results of blast assessments were analyzed on MEGA 6.00 software for alignment and Phylogenetic interference.

Results

Isolation of bacteria

A number of Cultivable bacteria on the basis of their morphological characteristics were isolated Out of these 13 were selected and labeled as; S-1 toS-13 for further studies, for the reason that of their ability to grow on higher concentrations of copper (Cu).

These were characterized by;

1. Morphological analysis
2. Biochemical analysis
3. Heavy metal resistance
4. Molecular analysis.
5. Phylogenetic analysis.

Morphological analysis

Pure isolates were examined for the five colony morphological features as described in (Cappuccino and Sherman (2005) laboratory manual. These five morphological features include form, edge, surface, elevation and color of colony.

Colony Morphology

Staining results

Gram staining was done to discriminate bacteria on the basis of biochemistry of cell wall. All the isolates were gram positive and spore forming.

Biochemical Analysis Results

Biochemical tests are used to label microorganisms on the basis of their metabolism. Following biochemical tests were carried out as described in laboratory manual of (Cappuccino and Sherman (2005).

The results are elaborated as; All biochemical test results;

Table 2. Copper resistance analysis results.

Isolates	Indole	Methyl Red	Voges-Proskauer:	Citrate utilization Test	Catalase	H_2S production	Motility
S-1	+	_	_	_	+	_	+
S-2	+	_	_	+	+	_	+
S-3	+	_	_	_	+	_	_
S-4	+	_	_	_	+	_	_
S-5	+	+	_	+	+	_	+
S-6	+	+	_	_	+	_	+
S-7	+	_	_	+	+	_	+
S-8	+	_	_	+	+	_	+
S-9	+	_	_	+	+	_	+
S-10	+	_	_	_	+	_	_
S-11	+	_	_	+	+	_	+
S-12	+	_	_	+	+	_	_
S-13	+	_	_	+	+	_	_

MIC(result)

All the isolates were resistant to Copper(Cu)at different concentration (50-800µg/ml). The resistance of isolates at different concentrations is shown in Table 3.

16s rRNA analysis and Amplification 1500bp segment of 16S rRNA gene was amplified with the primers 27f and RD1. PCR product on gel 16s rDNA amplification product is run on agarose gel (1.5 %), documented on Gel Doc system as shown in Fig 1.

Table 3. MIC for selected metals in µg/ml.

S#	1	2	3	4	5	6	7	8	9	10	11	12	13
Isolates	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
Copper resistance µg/ml	550	550	550	550	550	550	550	550	450	550	550	550	550

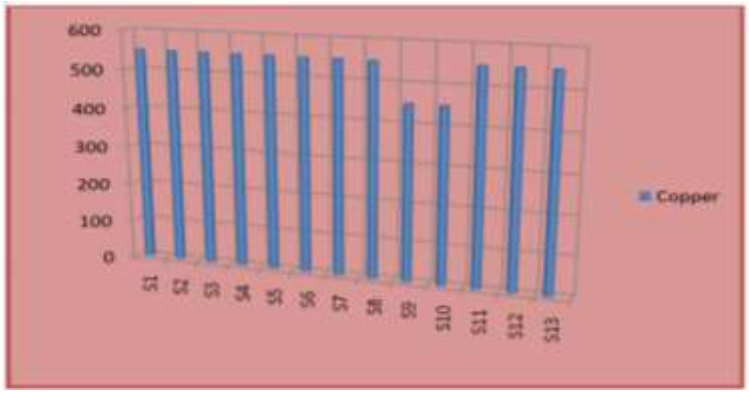

Fig. 1. Graph for MIC for selected metals in µg/ml (samples on y-axis, Heavy metal (cu) concentration in µg/ml on x-axis).

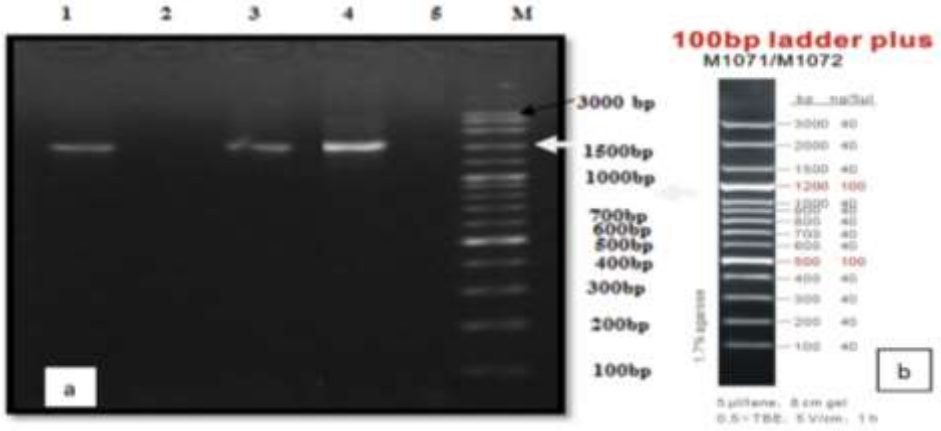

Fig. 2a. PCR Product on gel; PCR amplicons of bacterial isolates (Lane.M is ladder while well 1-3and4 are amplified products at 1500bp). The ladder was used of Fermentas of 3Kb. Length.: 2.b Fermentas 3kb Ladder for comparison.

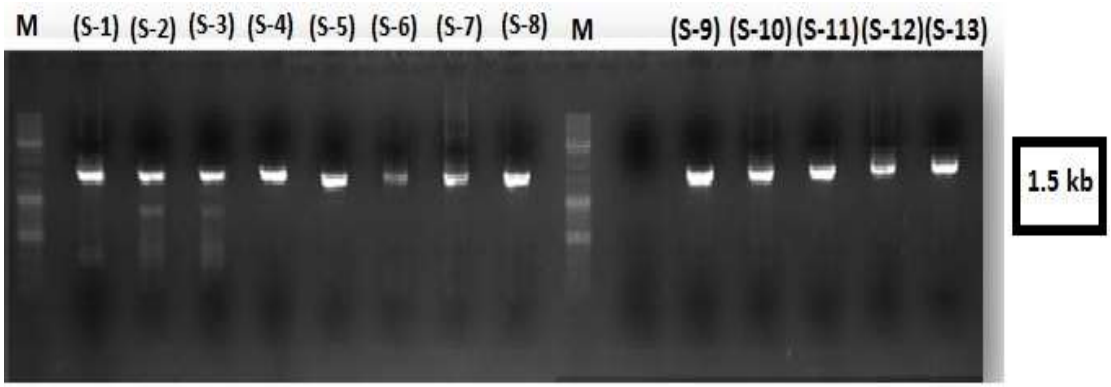

Fig. 3. (PCR amp icons of bacterial isolates (Lane.1 is ladder; from 2-8 is S1-S8, respectively; lane 9 is again ladder and lane 10-14 is S9-S13, respectively).

16S rDNA

Sequencing &Sequence analysis results 16S rRNA gene sequencing is used for the identification of cultivable and non-cultivable bacterial. Sequencing alignments by BLAST were noted down and displayed in the results.

Phylogenetic analysis results

Evolutionary relationships of taxa

The evolutionary history was indicated using. The Neighbor-Joining method. The percentage of duplicate trees in which the linked taxa are clustered together in the bootstrap test (100 replicates) is shown next to the branches.

The analysis involved 10 nucleotide sequences Evolutionary analyses and was done using MEGA6.00 (Tamura *et al.,* 2013).

Table 4. Summary of the identified isolates of sequence alignment.

#	Identical organisim	identity %	Lineage
S1	*Bacillus thuringiensis serovarkonkukian str.*	100%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
S2	*Bacillus weihenstephanensis* KBAB4	99%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
S4	*Micrococcus luteus NCTC 2665*	96%	Bacteria; Actinobacteria; Actinobacteriadae; Actinomycetales; Micrococcineae; Micrococcaceae; Micrococcus.
S5	*Bacillus anthracis str. Sterne chromosome*	100%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
S7	*Bacillus cereus* ATCC	100%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
S8	*Arthrobacterarilaitensis*RE117	98%	Bacteria; Actinobacteria; Actinobacteriadae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter.
S9	*Bacillus anthracis str. Ames*	100%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus Bacillus cereus group.
S11	*Bacillus pseudomycoides* DSM 12442	99%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group
S12	*Bacillus toyonensis*BCT-7112,	99%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
S13	*Bacillus subtilis* subsp. *subtilis str.*	100%	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.

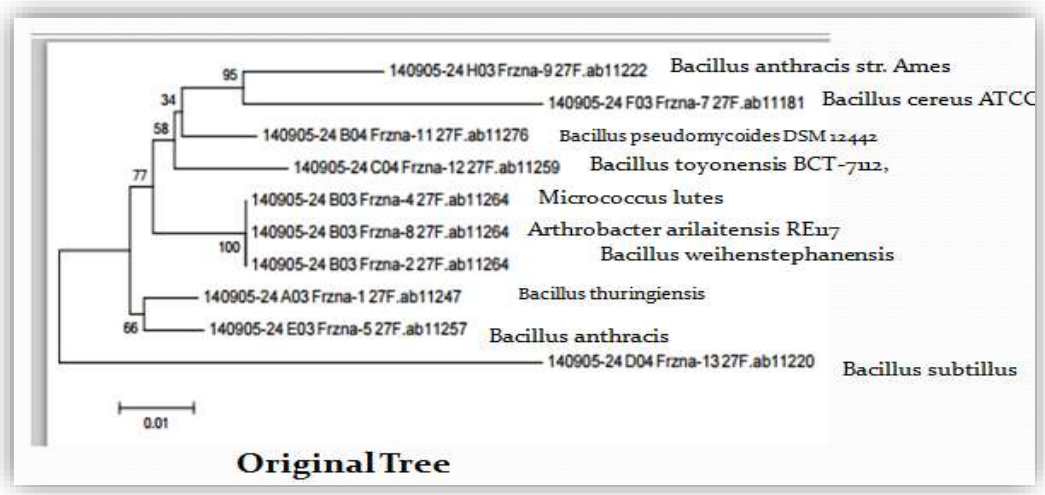

Fig. 4. Original trees with Bootstrap values constructed via MEGA-7.

Discussion

Although the heavy metals are toxic to microorganisms as to the other living organisms but a number of microorganisms can adopt certain mechanisms to become resistant and can be potentially applied to the polluted sites of the environments (Jiang *et al.,* 2008).

In this study 13 isolates identified were highly resistant as they grew on high concentration of copper. As most of our isolates MIC on media supplemented with copper recorded up to 650µg/ml. As mentioned in results that most of the isolates were negative for MRVP and H_2S production while the majority were found positive for citrase, Catalase, motility and Indole tests, these results are somehow typical for members of *Bacillus* species. Morphological and biochemical analysis were helpful just to get maximum numbers of pure cultures but cannot be considered as a reliable method. 16S rRNA technique was performed in order to get reliable results. The obtained sequences were BLAST for the homology with the existing sequences on the database. As a result the 10 out of 13 isolates were sequenced well and aligned which were S1, S2. S4. S5, S7, S8, S9, S11, S12, S13. All were similar to bacillus except S8 and S4 which were identical to genera *Arthrobacter* and *micrococcus* respectively. Among the bacillus majority of the isolates (S1, S2, S5, S7, S9, S11, and S12) sequence met with the bacillus cereus group lineage, these results were same in Phylogenetic analysis.

Bacillus cereus is a spore-forming, motile, aerobic rod shaped bacteria which is also able to grow in the aerobic conditions. In gram staining B. *cereus* are gram negative.S1 is of great importance as *Bacillus thuringiensis* has a distinctive property of producing CRY protein which is used as an bio-insecticide (Battisti *et al.,* 1985). Specific gene encoding this protein is inserted in crops in order to produce insecticides, BT cotton (transgenic cotton) is an example. So this isolate may be used in future to develop insect resistance in plats/crops.

The *B. thuringiensis* is reported to generate entero-toxins and responsible for food poisoning. As spraying of this organism to save crops against insect attack has become common in a number of countries, to declare protected spraying with *B. thuringiensis*, the organism in use should be incapable to turn out food poisoning toxin (Arnesen *et al.,* 2008, Granum and Lund 1997).

S8 which is *Arthrobacter arilaitensis* has its importance in biotechnology, as it is one of the major bacterial species found at the surface of cheeses, especially in smear-ripened cheeses, where it contributes for its specific color, flavor and texture properties of the final product.

Most of the eukaryotes and prokaryotes are capable of bio sorption because of cell wall, where the functional groups are present with metal binding capacity. Sometimes these metals may be deposited in the cytoplasm but mostly these are reported to be attached to the cell wall. Copper is an important metal with the high capability to accumulate in cell wall of prokaryotes when applied to bioremediation of polluted sites (Wei *et al.,* 2009). Bacillus species include psychophysics to thermophilic microorganisms, which lead them to settle a wide variety of environments. Same to other species of Group bacillus, bacillus cerese species are metabolically different that make them able to survive in different environmental conditions (Guinebretiere *et al.,* 2013). That's why our isolates survived in the severe conditions of coal mines of Sorrange Balochistan.

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