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16s RNA Partial Sequencing of Isolated Strains of Metal Resistant Bacteria

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ABSTRACT: Four multi-heavy metal resistant strains of bacteria were isolated from industrial effluent rich in heavy metal contaminants. Partial sequences of the 16s rRNA genes of the isolated bacterial strains were identified following the isolation and cloning of the required 16s rRNA region using PCR amplification. The resulting sequences were aligned and sequenced with the aid of ABI Prism 377 automatic sequencer. The evolutionary distances and the phylogenetic trees were inferred and analyzed by using maximum-composite-likelihood method and neighbor- joining method respectively. The bacterial strains identified were similar to natural strains of *Bacillus pumilus, Bacillus subtilis, Pseudomonas aeruginosa, Serratia liquefacians* respectively but with multi- heavy metal resistant ability. They were seems to resistant against Cu, Cr, Ni, Pb, Mn and Zn. The overall average level of sequence similarity between the isolated strains and their matched counterparts is found to be 99.98 %.

Keywords: Heavy metal reducing bacteria, *Bacillus subtilis, Pseudomonas aeruginosa, Bacillus pumilus*, *Serratia liquefaciens*

I. INTRODUCTION

The toxicity caused due to heavy metals is a serious problem all over the world as they cause significant damage to human health. Heavy metals are the main category of pollutants in water bodies, ground, industrial and also treated wastewater (Valdman *et al.* 2001). Toxic heavy metals are found in discharged waste water effluents from industries like electroplating, steel, motor vehicles, alloy, motor vehicles, paint, chemicals, pigment, textile, aircraft etc.,(Saithong & Prasertsan 2002; Sani *et al.* 2001). Cadmium (Cd), Nickel (Ni) and lead (Pb) are the most commonly released heavy metals into the environment (Aksu, 1998; Patterson, 1977; Doenmez and Aksu, 1999). Trace amounts of heavy metals are quite necessary as co-factors of enzymatic reactions, but when present in high levels, these heavy metals may cause extreme toxicity to living organisms owing to the inhibition of metabolic reactions (Rai *et al.,* 1981; Macaskie and Dean, 1989). The microbial flora responds to these heavy metals by several processes; including the transport across the cell membranes, bio-sorption to the cell walls and entrapment in extracellular capsules, precipitation, complexation and redox reactions (Huang *et al.,* 1990; Brady *et al.,* 1994; Brady and Duncan, 1994; Veglio *et al.,* 1997).

The conventional methods like precipitation, redox reactions, ion exchange, membrane filtration and evaporation are capable of removing these toxic heavy metals from the environment, However, these methods are extremely expensive and inefficient for metal removal from dilute solutions of the range 1 to 100 mg of dissolved metal per liter (Volesky 1990). The use of microbial communities to assess the impact caused by anthropogenic stress in natural habitats is increasing; however, there is considerable debate as to which approach is the most useful. Hence, micro flora is considered to provide alternative means to conventional methods of metal removal and recovery (Ozdemir *et al.,* 2004).

Microbial collection of bacteria, fungi and algae, both in live and inactivated form are reported to be capable of removing hazardous heavy metal ions by two well known process mechanisms i.e., (i) biosorption: binding of metal ions to cell walls devoid of energy dependency and (ii) bioaccumulation: an energy- dependent process of metal uptake into the cells (Karna *et al*. 1996; Volesky 1994; Li *et al.* 2004). They can be single metal resistant or multi- heavy metal resistant depending on their genomic sequence variability. A single bacterial strain can be found to be resistant to many metals.

As reported by Cànovas *et al.,* 2003, the genome sequence of *P. putida* KT2440 contains 61 open reading frames involved in tolerance and resistance to a range of metals (Cànovas *et al.,* 2003). A bacterium isolated on the basis of tributyltin resistance was found to be resistant to multiple metals including Hg, Cd, Zn, Sn, Cu, and Pb (Pain and Cooney, 1998).

The present study involves the characterization of four strains of bacterium isolated from heavy metal rich industrial effluent with multi- heavy metal resistance capacity. The strains are further classified using phylogenetic tree construction with the aid of maximum-composite-likelihood method and neighbor-joining method.

II. MATERIALS AND METHODS

A. Sample isolation and preparation

Several bacterial strains were isolated from the industrial effluent rich in heavy metals and in which four strains were used for the study. The bacterial strains with heavy metal resistant capability for copper, zinc, lead, nickel chromium, manganese were prepared for DNA extractions and further research is subjected to 16s rRNA partial sequencing to determine their respective family of similarity (Edita *et al.,* 2011).

B. DNA isolation procedure

DNA extraction was performed using 2-mL of the isolated bacterial strains, collected at the mid-exponential growth phase using the Roche Kit (Germany) according to the manufacturer's instructions.

Primers: Universal set of the Eubac primers as mentioned below were used for the study.

27F - 5′- AGAG TTTG ATCM TGGC TCAG -3′ (forward primer)

1492R - 5′- GGTT ACCT TGTT ACGA CTT -3′ (reverse primer)

PCR amplification: The PCR was performed on a thermal cycler (Eppendorf) with 50µl reaction mix. The reaction mixture contained 10x amplification buffer (5µl), 1.5mM $MgCl_2$ (5µl), 1µl of each forward and reverse primer, 1µl dNTP and 0.25µl Taq polymerase. After an initial denaturation at 95°C for 1 min, amplification was carried out with 35 cycles of 35s denaturation step at 94°C, annealing for 40s at 55°C, extension for 2 min at 72°C followed by a final extension for 8 min at 72 °C. The PCR products were analyzed by electrophoresis using 1.2% agarose gel (Genei).

C. DNA Sequencer

The PCR product was purified using the Qiagen PCR purification kit and then sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA).

D. Phylogenetic tree reconstruction

The evolutionary distances were computed using the maximum-composite-likelihood method (Tamura *et al.,* 2004) and the evolutionary analyses was conducted using MEGA7 software (Kumar *et al.,* 2016). The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method. Based on the phylogenetic tree constructs, similarity index was generated and compared with known sequences against GenBank database.

III. RESULTS

Based on the comparative analysis of the sequences with already available database entries showed that the strains RHMR2, RHMR7 were close to the members of the genus *Bacillus* and RHMR14, RHMR21 were similar to *Pseudomonas* and *Serratia* respectively. The sequence similarity and phylogeny clearly indicates the following:

RHMR2 is a strain of *Bacillus pumilus* (Fig. 1)

RHMR7 is a strain of *Bacllus subtilis* (Fig. 2)

RHMR14 is a strain of *Pseudomonas aeruginosa* (Fig. 3) and

RHMR21 is a strain of *Serratia liquefacians* (Fig. 4)

16S rRNA sequences of the mentioned bacterial strains were submitted in the GenBank database under the following accession numbers:

BankIt2039777 Seq1 MF662817

BankIt2039869 Seq2 MF662818

BankIt2039874 Seq1 MF662819

BankIt2039875 Seq1 MF662820

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei., 1987). The optimal tree with the sum of branch length of the four strains is shown in Table 1.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

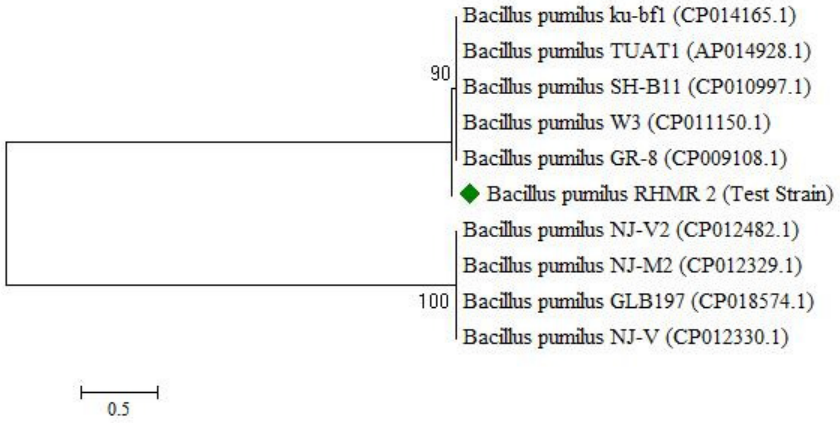

Fig. 1. Phylogenetic tree of *Bacillus pumilus* RHMR 2.

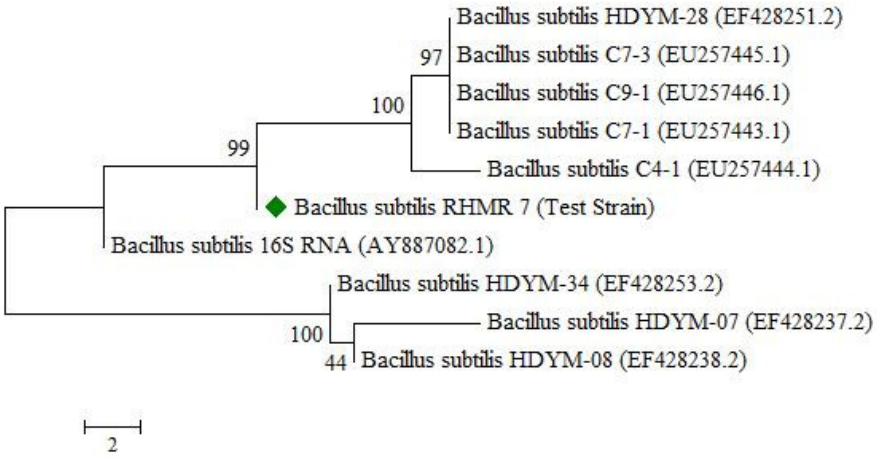

Fig. 2. Phylogenetic tree of *Bacillus subtilis* RHMR 7.

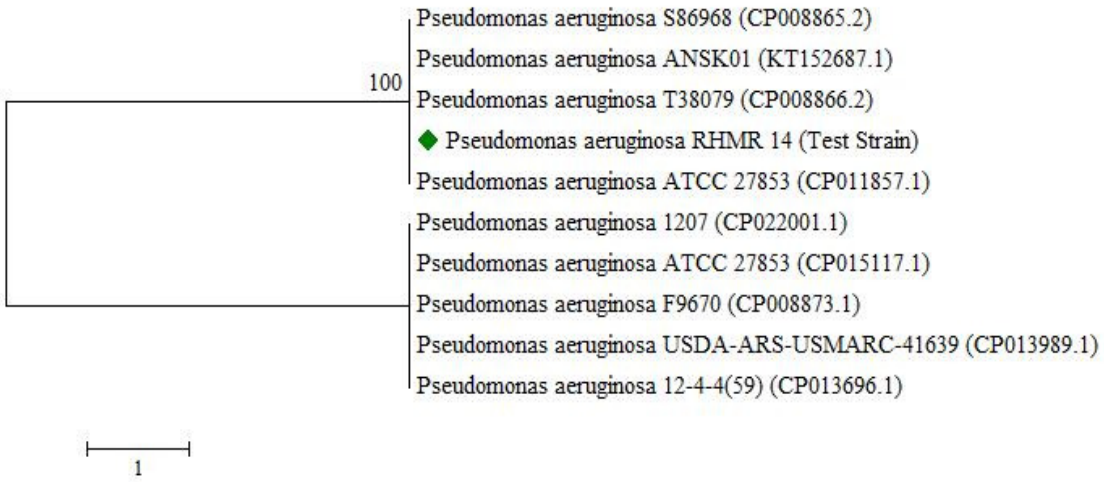

Fig. 3. Phylogenetic tree of *Pseudomonas aeruginosa* RHMR 14.

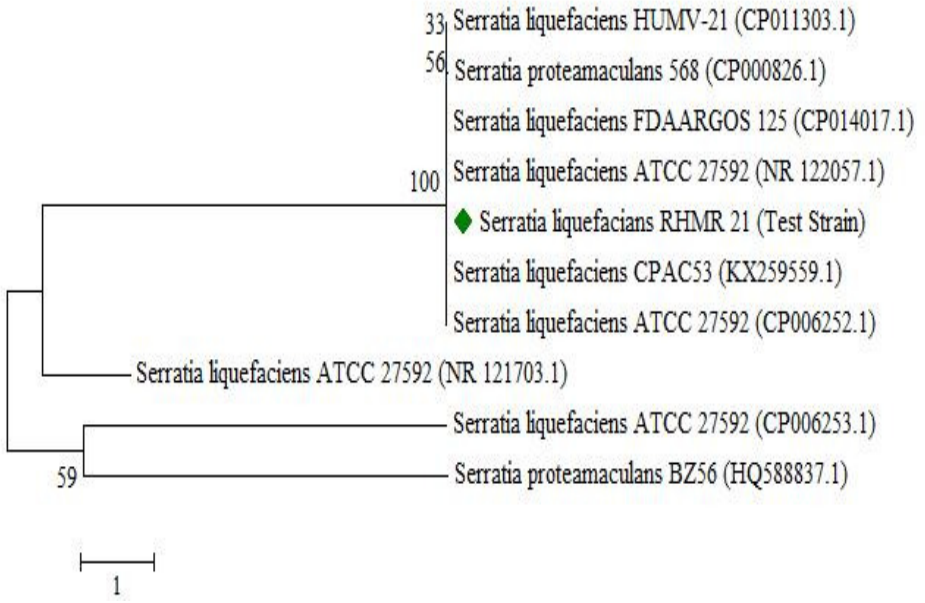

Fig. 4. Phylogenetic tree of *Serratia liquefaciens* RHMR 21.

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1517 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher and Tamura, 2016). The percentage of sequence similarity between the isolated strains and their matched counterparts is mentioned in Table 2.

In figure 1, the bacterial strain *Bacillus pumilus* RHMR 2 was depicted to be most similar and found a place between, *Bacillus pumilus* GR- 8 and *Bacillus pumilus* NJ- V2 respectively with 99.93 % sequence similarity.

In figure 2, online homology search depicted that the 16S rRNA sequence of the strain *Bacillus subtilis* RHMR 7 was found to be the most similar to the sequence of *Bacillus* at 100 % similarity to the established species.

In figure 3, the 16S rRNA sequence of *Pseudomonas aeruginosa* RHMR 14 was found to be totally in sync with the already established Pseudomonas strains by 100 % similarity.

In figure 4, the sequence strain of *Serratialiquefaciens*RHMR21 was found to be 100 % similar to the established *Serratia* species on the basis of genus name.

Table 1: Optimal tree with the sum of branch length.

Sequence name	Optimal tree with the sum of branch length
Bacillus pumilus RHMR2	5.80162063
Bacillus subtilis RHMR7	21.19581977
Pseudomonas aeruginosa RHMR14	7.90492661
Serratia liquefaciens RHMR21	17.99600040

Table 2: Percentage of sequence similarity.

Sequence name	Percentage of similarity (%)
Bacillus pumilus RHMR2	99.93
Bacillus subtilis RHMR7	100.00
Pseudomonas aeruginosa RHMR14	100.00
Serratia liquefaciens RHMR21	100.00

IV. DISCUSSION

In this paper, we report the 16S rRNA partial sequencing characterization of four bacterial strains isolated from different industrial effluents rich in heavy metal debris Cu, Zn, Pb, Ni, Mn and Zn,. The strains were found to be closest in character to the aforementioned similarities with multi heavy metal resistant capability against Cu, Mn, Zn, Pb, Ni, Zn. The results indicate the percentage of similarity with their established counterparts and their overall similarity is found to be 99.8%. In addition to their close resemblance to the original strains, they also exhibit heavy metal resistance ability and hence can be used for potential heavy metal treatment studies.

The culture medium choice appears to be very significant for a survey of the structure and variety of the culturable part of the bacterial community. This is due to the fact that significant differences observed in the occurrence of the growing isolates on both culture media and their circulation into broad taxonomic groups. It is obvious that MM generates suitable conditions for the growth of a wide variety of natural bacterial isolates than RM does, indicating that MM offers conditions more similar to those found in the environmental sample than is offered by RM. Moreover, these results also indicate that the use of both types of culture media provided a better picture of the structure and diversity of the culturable part of the microbial communities, because this approach allows the cultivation of bacterial strains with different growth requirements, enabling a variety of natural bacteria to be identified than would be possible using only one type of medium. However, the significance of a nutritionally poor medium for the cultivation and isolation of unculturable microorganisms must be emphasized. Conventional culture media are nutritionally rich and their use for the cultivation and isolation of microorganisms from environmental samples renders the majority of microorganisms unculturable (Joseph *et al*. 2003; Edita *et al.,* 2011).

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