Growth media composition and heavy metal tolerance behaviour of bacteria characterized from the sub-surface soil of uranium rich ore bearing site of Domiasiat in Meghalaya

Rakshak Kumar¹, Macmillan Nongkhlaw¹, Celin Acharya² and S R Joshi¹*

¹Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North-Eastern Hill University Umshing, Shillong 793 022, India
²Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

Enrichment based cultivation method was utilised to isolate uranium (U)-tolerant bacteria from the sub-surface soil of U-rich ore bearing site of Domiasiat in Meghalaya, India. Two isolates with distinct colony morphology and pigmentation were closely matched as Serratia marcescens PKRS1 and Pseudomonas ficuserectae PKRS11 based on 16S rRNA gene sequence similarity analysis and phylogenetic clustering approach. Mueller-Hinton (MH) and Low phosphate (LP) medium were used for comparative determination of the minimum inhibitory concentration (MIC) of U and other metals against the identified bacteria. The wild isolates tolerated higher amount of U and other metals when compared to the type strain S. marcescens ATCC13880. Higher MIC was observed in MH than LP medium. The present study reveals that the metal-microbe interaction, such as, uptake and precipitation, are highly influenced by growth media composition. LP medium as the basal medium offers more authentic results due to low carbon source and negligible phosphates, which provides lower precipitation affinity to metals making it uniformly available to the isolates during growth as compared to complex MH medium.

Keywords: Domiasiat, low phosphate medium, minimum inhibitory concentration, Mueller-Hinton medium, uranium tolerant bacteria

Introduction

India possesses pockets of uranium (U) deposits under its earth’s crust and one such deposits includes the phanerozoic sandstone type deposits of Kylleng-Pyndengsohiong (popularly known as Domiasiat and Wahkhlyn) in the West Khasi Hills district of Meghalaya. The deposits form a part of Cretaceous Mahadek basin containing 9.22 million tonnes of ore reserves of an average ore grade around 0.1% U₃O₈ along the Killung, Rangam, Umla, Pyrnotbri, Jimrey, and Tyrkhang blocks, covering 10 km² area in and around Domiasiat and Phladiloin¹². In the year 1992, test recovery plant was set up by the Atomic Mineral Directorate (AMD) for Exploration and Research, India and in 1993 the U deposits were evaluated and estimated (available online at www.amd.gov.in/about/milest). Detailed radiometric surveys during 1983-84 by the AMD discovered the Killung block as the radioactive zones along the Phot Killung Nala¹³.

Diverse form of bacteria exist in such extreme deep subsurface radionuclides/metal enriched reserves as the one mentioned above and these bacteria are known to possess the characteristics of metal resistance⁴⁻⁸. Earlier reports have suggested that these indigenous bacteria, which grow in condition with high concentration of metals, must maintain a homeostasis within the cell that keeps the reactive heavy metals at an optimal, sub-toxic level⁹. Resistance factors may allow them to maintain intracellular low levels of heavy metals or intracellular fractionation of the metal in non-harmful complexes¹⁰. Thus, adapted microbial populations of metal rich areas are prone to show higher resistance to heavy metals as compared to populations of non-contaminated sites⁵. The resistant bacteria adapted to such heavy metal rich environments has its importance in biosorption, bioprecipitation, extracellular sequestration, transport mechanisms and/or chelation¹¹, and these resistance mechanisms may act as basis for the use of microorganisms in bioremediation approaches.

In the present study, enrichment based cultivation method was used to isolate U-tolerant bacteria. Two of the morphologically distinct isolated bacteria
were identified and were tested for their tolerance against U and multi-metal using two growth media. The minimum inhibitory concentrations of metals supplemented in the growth media were analysed to assess if media composition influenced the metal tolerance behaviour of the bacteria.

Materials and Methods

Sampling Site

Samples were collected from sites surrounding the U exploratory drilling and test recovery plant of Domiasiat (25°30’N 91°30’E) located 130 km southwest of Shillong (capital city of Meghalaya) in Northeast India. It forms a part of Cretaceous Mahadek basin covering roughly an area of 13000 km². It is one of the largest sandstone-type U deposits in India containing 9.22 million tonnes of ore reserves with an average ore grade around 0.1% U₃O₈. The pH of the soil was acidic within the range of 4.3-6.3.

Screening and Isolation of Subsurface U-tolerant Bacteria

Sub-surface soils (15 cm deep to the top layer of the soils) were collected from the sites and duplicate soil samples (10 g) were inoculated in 100 mL of tryptone soy broth (diluted at 0.5%) (HiMedia, India) (pH 7) in Erlenmeyer flasks amended with 1 mM U(VI) as [UO₂(NO₃)₂.6H₂O] and incubated at 30°C at 150 rpm for 24 h. U-tolerant bacterial population were isolated by inoculating serial 10-fold dilutions of the enrichment cultures onto the tryptone soy agar (diluted at 0.5%) (HiMedia, India) plates supplemented with 1 mM U. Plates were incubated at 30°C for 72 h. Two colonies of morphologically distinct U-tolerant bacteria were picked up from the plates. Purity of the cultures were confirmed by streak plate method using nutrient agar medium and the cultures were preserved using 15% glycerol.

Isolation of Genomic DNA

Genomic DNA was extracted using the general protocol for bacteria as described by Ausubel et al with some modifications. Live cells of isolated pure culture were resuspended in 567 µL Tris-EDTA buffer, pH 8.0 (10 mM Tris, 0.1 mM EDTA). To this, 30 µL of 10% SDS and 2.4 µL of Proteinase-K (from a 25 mg mL⁻¹ freshly prepared stock) were added, mixed thoroughly and incubated at 37°C for 1 h. To the lysed cells, 100 µL of 5 M NaCl was added and mixed, followed by addition of 80 µL CTAB/NaCl solution. The mixture was incubated at 65°C for 10 min and then equal volume of chloroform-isoamyl alcohol (24:1) (~750 µL) was added and mixed gently (~10/s/tube). The aqueous phase (supernatant) was collected after centrifugation for 5 min at 13,000 rpm. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was then added to the aqueous phase and mixed gently (~10/s/tube). The tubes were centrifuged for 5 min at 13,000 rpm and 0.6 volume isopropanol was added to the separated supernatant to precipitate nucleic acids (mixed from top to bottom to obtain a stringy whitish DNA precipitate). Genomic DNA was spooled gently, washed with 70% ethanol, dried in air and dissolved in 10 mM Tris, 0.1 mM EDTA (TE) buffer, pH 8.0 at approx 0.5 µg µL⁻¹ concentrations. The purity of DNA was ascertained by OD₂₆₀nm/OD₂₈₀nm ratio, which was around 1.5 for the preparations.

Amplification and Sequencing of 16S rRNA Gene

The 16S rRNA gene sequences were amplified by PCR using two general bacterial 16S rRNA gene primers. PCR mixtures (25 µL) contained approx 30 ng of template DNA, 2 µM forward primer 27F, 2 µM reverse primer 1492R, 1.5 mM of MgCl₂ (Taq Buffer), deoxynucleoside triphosphates (250 µM each of dATP, dCTP, dGTP and dTTP) and 0.6 U of Taq polymerase. DNA amplification was carried out in Gene AMP PCR system 9700 (Applied Biosystems, USA) with an initial denaturation step of 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, and then a final extension step of 72°C for 5 min. Approx 1500 nucleotides were amplified using PCR. Templates replaced with sterile water were always used as negative controls.

For sequencing, the amplified 16S rRNA gene products were purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany). The purified PCR products were bi-directionally sequenced using the forward, reverse and internal primers corresponding to Escherichia coli positions 357F, 926F, 685R and 1100R. Sequencing was performed using Big Dye Terminator cycle sequencing kit v.3.1 (Applied Biosystems, USA) with an automated Genetic Analyzer ABI 3130XL (Applied Biosystems, USA). Sequencing reaction was performed with 20 µL reaction mixtures containing template DNA (approx 50 ng of 16S rRNA gene PCR product) and 1 pmol of sequencing primers along with the ABI Ready Reaction Mix consisting of deoxynucleotides (ddNTPs) tagged to fluorescent dyes, dNTPs, Ampli Taq DNA polymerase, MgCl₂ and ABI Ready Reaction buffer. The PCR thermal cycler was
programmed for 30 cycles, with initial denaturation at 96°C for 5 min, followed by each cycle consisting of a denaturation step at 96°C for 10 sec, annealing at 55°C for 10 sec and extension at 60°C for 4 min. Post reaction cleanup and resuspension was performed for removal of unincorporated dye terminators from the sequencing reaction using 125 mM EDTA, 3M sodium acetate and ethanol. The ethanol precipitated dried pellet was mixed with 10 µL of Hi-Di formamide, heated at 94°C for 2 min for denaturation and quickly chilled on ice. Aliquots (10 µL) of the sample were loaded onto the automated Genetic Analyzer.

**Phylogenetic Analyses**

The phylogenetic neighbors were obtained using the Basic Local Alignment Search Tool (BLAST)\(^1\) program against the database of type strains with validly published prokaryotic names (available online: http://www.eztaxon.org)\(^15\) for each of the selected isolates. Molecular Evolutionary Genetics Analysis software (MEGA version 4) was used for phylogenetic analyses\(^16\) for both the phylogenetic tree. The sequences of identified phylogenetic neighbors were aligned with the sequences of isolates using ClustalW inbuilt with MEGA 4. *Deinococcus radiodurans* M21413 was used as the outgroup organism for both the tree. Neighbor-joining method was employed to construct the phylogenetic tree with 1000 bootstrap replications to assess nodal support in the tree. The isolates were assigned to a species based on the high similarity percentage (>98.7%) and clear phylogenetic clustering.

**Determination of Minimum Inhibitory Concentration (MIC) with Mueller-Hinton (MH) Medium**

Analytical grade salts of uranyl nitrate [UO\(_2\)(NO\(_3\))\(_2\)·6H\(_2\)O] (Merck); copper sulphate (CuSO\(_4\)·5H\(_2\)O), cadmium nitrate [Cd(NO\(_3\))\(_2\)·5H\(_2\)O] and lead nitrate [Pb(NO\(_3\))\(_2\)] (HiMedia), were used to prepare the stock solutions and U solution was stored in the dark to prevent photodecomposition. Stock solutions of metals were filter sterilized through a 0.22 µm nitrocellulose membrane filter (Millipore, India). The selected isolates including the type strain *Serratia marcescens* ATCC13880 were grown to mid-exponential phase in MH broth and 10 µL of the cell suspension were spotted onto MH agar plates (g L\(^{-1}\)): Beef infusion, 300.0; casein acid hydrolysate, 17.50; starch, 1.50 (HiMedia, India), supplemented with metal concentrations in increasing concentration (by a factor of two). The pH of the medium was adjusted to 7.0 prior to inoculation. Growth was recorded after 72 h of incubation at 30°C\(^17\). The lowest concentration of metals which completely inhibited the visible growth of the test strains on the plates was considered as the MIC.

**Determination of MIC with Low Phosphate (LP) Medium**

Both the isolates and the type strain were grown to mid-exponential phase in LPM broth which contained (g L\(^{-1}\)): Tris, 14.5; NaCl, 4.68; KCl, 1.5; NH\(_4\)Cl, 1.0; glycerol, 5; Na\(_2\)SO\(_4\), 0.043; CaCl\(_2\), 0.03 (pH adjusted to 7.5 with concentrated HCl)\(^18\). The cells were then washed twice with 0.9% NaCl, and 10 µL of the cell suspension were spotted onto LP agar medium plates (150 mm diam)\(^19\) for their tolerance in increasing (by a factor of two) concentration of metals\(^20\). The MIC was expressed as the metal concentration that inhibited confluent growth of the spotted culture after 2 d incubation at 30°C\(^21\).

**Nucleotide Sequence Accessions Numbers**

The accession numbers obtained from NCBI Genebank for the 16S rRNA gene sequences of bacterial isolates PKRS1 (1494 bp) and PKRS11 (1405 bp) were GU270569 and HM747952, respectively.

**Results and Discussion**

**Closest Match of Selected Bacteria**

16S rRNA gene sequences analyses were performed to establish the identity of two U-tolerant bacteria obtained from the U-supplemented tryptone soy agar plates. More than 1400 base pairs of the two selected isolates were generated and used to perform BLAST program against the database of type strains at EzTaxon server to identify the nearest phylogenetic neighbours. The 1494 bp 16S rRNA gene isolate PKRS1 produced nearest homology of 99.7% similarity with 16S rRNA gene sequences of *S. marcescens* KRED(T) and the 1405 bp 16S rRNA gene isolate PKRS11 showed similarity of 98.85% with the nearest homolog *Pseudomonas fuscicata* JCM 2400(T). Consequently, sequences with high similarity scores were retrieved for each isolates and two phylogenetic trees were created using MEGA (version 4). Other members of the family *Enterobacteriaceae* and *Pseudomonadaceae*, those displayed close sequence similarity were also included to study their relatedness with the representative isolates. Two major clusters were obtained for isolate PKRS1 and the isolate clustered together with *S. marcescens* KRED(T) with a good
bootstrap support of 93% (Fig. 1). In case of the isolate PKRS11, two major clusters were obtained and the isolate clustered together with \( P. \) \textit{ficuserectae} JCM 2400T with a good bootstrap support of 93% (Fig. 2). Hence, \( S. \) \textit{marcescens} is established as the closest match of isolate PKRS1 and \( P. \) \textit{ficuserectae} as that of the isolate PKRS11.

**U and Multimetal Tolerance using MH and LP Medium**

The study on MIC of metals for the growth of selected \( S. \) \textit{marcescens} PKRS1 and \( P. \) \textit{ficuserectae} PKRS11 showed significant tolerance of isolates to U and other heavy metals, viz., cadmium, zinc, copper and lead, when compared with the MIC of the type strain \( S. \) \textit{marcescens} ATCC13880 (Fig. 3). It was observed that the MIC was higher in MH agar assay as compared to the LP agar assay. Physical observation during media preparation revealed no precipitation of metals in basal LP medium, while clear precipitates of metals especially in case of lead and U were observed in complex MH medium. Distinct variations in the MIC values were observed even after similar conditions were provided in both the assays, except for the composition of media. Moreover, it is theorized before that MIC values varies with the type of media used. Due to different conditions of diffusion, complexation and availability of metals, higher MIC is reported in solid media in comparison to liquid media; and in rich media in comparison to minimal media. To propose the identified bacteria as superior in their metal tolerance based on the MIC results, when compared to other such metal tolerance studies, is often difficult as the condition used differ in different investigations. However, the MIC study performed in the present study clearly indicates the higher tolerance capacity of the natural isolates as compared to the MIC values obtained for type strains performed under the same laboratory conditions. These high MIC results were coherent with the findings of other investigators, indicating the increased occurrence of metal tolerant bacteria with the increase of heavy metals/radionuclide concentrations in metal contaminated sites.

The higher MIC values displayed in MH may be attributed to the non-uniform availability of metals in the medium due to metals affinity for precipitation in rich media. There was no observed precipitation of the used metals in LPM, which may be due to the

---

**Fig. 1**— Neighbor-joining tree based on 1494 bp of 16S rRNA gene sequences of isolate PKRS1 depicting the phylogenetic relationships of PKRS1 with the related species obtained from the database of type strains with validly published prokaryotic names at EzTaxon server. \( D. \) \textit{radiodurans} M21413 was taken as the outgroup organism. The scale bar corresponds to the expected number of changes per nucleotide position.

**Fig. 2**— Neighbor-joining tree based on 1405 bp of 16S rRNA gene sequences of isolate PKRS11 depicting the phylogenetic relationships of PKRS11 with the related species obtained from EzTaxon server (the database of type strains). \( D. \) \textit{radiodurans} M21413 was taken as the outgroup organism and the scale bar corresponds to the expected number of changes per nucleotide position.

**Fig. 3**— Minimum inhibitory concentration (MIC) determination of the bacterial isolates PKRS1, PKRS11 and the type strain \( S. \) \textit{marcescens} ATCC13880 against uranium (U), copper (Cu), cadmium (Cd) and lead (Pb). Parentheses indicates the growth media utilised; MH= Mueller-Hinton medium, LPM= Low phosphate medium.
presence of very low carbon and negligible phosphate source. On the basis of the present study, we propose that the metal-microbe interaction mechanisms, such as, uptake and precipitation, can be more authentically studied using the basal LPM as this contains negligible phosphates and carbon source and thus lowering the metal’s precipitation affinity and enhancing their availability to the isolates. The same is not true for a complex medium like MH as the metals get complexed with the medium ingredients. This makes the metals negligibly available to the growing isolates, giving a false indication of higher tolerance behaviour by the bacteria in complex medium.

Acknowledgement
RK and SRJ acknowledge the financial support received from BARC-BRNS, Department of Atomic Energy, Government of India, Mumbai, and MN acknowledge the financial support received from the Department of Biotechnology, Government of India, New Delhi for carrying out the present study.

References