International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **2(12),** 102-106, December **(2013)** *Int. Res. J. Biological Sci.*

Isolation, Identification and Characterization of *Curtobacterium* sp. YU-SS-C-67 for phosphate Solubilization and Uranium Tolerance

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Available online at: www.isca.in, www.isca.me
Received 15th August 2013, revised 22nd September 2013, accepted 24th October 2013

Abstract

Management of nuclear waste particularly uranium is of great environmental concern. Bioremediation of uranium using bacteria offers a less expensive, in situ alternative to the commonly used physico-chemical techniques. Recent bioremediation studies on heavy metals have focused on bioprecipitation as metal phosphates. In this respect, the present study deals with the isolation and characterization of a phosphate solubilizing Curtobacterium sp. YU-SS-C-67 from the vicinity of a proposed uranium mining site, Gogi (Karnataka, India). Following bacterial growth in the Pikovskaya's broth, 271.13 mgL^{-1} of phosphate was solubilized from insoluble tri-calcium phosphate with the drop in the media pH from 6.93 to 5.8. When tested for uranium sensitivity, the bacterium showed 12.89% reduction in cell number which was significantly lower ($p < 0.01$) compared to 33.21% reduction seen in the reference strain Escherichia coli ATCC 25922^{T}. These results indicate that the isolate Curtobacterium sp. YU-SS-C-67 having the ability to solubilize phosphate as well as tolerate the chemical toxicity of uranium can find application in bioremediation technology. Further studies are demanded on isolation of microbial communities from these environments which may harbor interesting candidates for biological based remediation of uranium and other heavy metals.

Keywords: Phosphate solubilizing bacteria, uranium, radionuclide, *Curtobacterium*, biomineralization, bioremediation.

Introduction

Contamination of soil and groundwater with uranium (U) has significantly increased due to various anthropogenic activities[1]. Owing to the toxicity of this element on human health and ecosystem, investigations into the long term management of these radionuclides are of great environmental concern[2]. The physico-chemical remediation methods are often expensive and generate secondary waste streams[3, 4]. Hence, biological based treatment of U has evoked considerable interest as eco-friendly and economically attractive strategy particularly in case of low grade contamination[5, 6].

Uranium exists in the environment primarily as an insoluble tetravalent uraninite mineral or as soluble hexavalent uranyl ion[7]. Most of the bioremediation studies on U have focused on lowering its solubility by reduction of U (VI) to U (IV) thereby confining the element from being mobile[3, 8]. But, bioreduction can be only applied to the anaerobic system as U (IV) gets reoxidized into U (VI) on the availability of oxygen[9]. Hence, recent bioremediation methods have shifted the focus on biomineralization of U into stable uranyl phosphate minerals. Since the availability of free phosphate in the environment is limited, phosphate solubilizing bacteria can be employed to increase the free phosphate thereby inducing the precipitation of heavy metals and radionuclides[10]. In addition, with plant growth promoting qualities, these phosphate solubilizing bacteria can also assist in phytoremediation of metal contaminated sites[11-13]. But the applicability of these bacteria in U remediation depends on their potential to tolerate the chemical toxicity of uranium.

It is known that the environments heavily polluted with metals and radionuclides are rich sources of metal resistant and/or accumulating bacterial strains[14, 15, 16]. In this respect, the present work deals with the isolation and characterization of a bacterium YU-SS-C-67 from a tube well water collected from Gogi located at the Bhima river belt of Karnataka. Having both the abilities of phosphate solubilization and U tolerance, the isolate can offer to be a candidate in the development of bioremediation technology for uranium.

Material and Methods

Isolation of bacteria: The bacterium YU-SS-C-67 was isolated from a water sample collected from a tube well of Gogi village in Yadagiri district (Karnataka, India). The sample was serially diluted and plated on Pikovskaya's agar media[17] and plates were incubated at 32 ± 2°C for 72 hours. The pure isolate designated as YU-SS-C-67 was preserved in 30% (v/v) glycerol at -80^{0}C for further analyses.

Determination of phosphate solubilization ability: Solubilization Index: Solubilization Index is described as the ability of the bacteria to solubilize insoluble phosphate which is calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter[18]. For this, the bacterium was plated on

Pikovskaya's agar media, plates were incubated at 32 ± 2°C and were analyzed for the zone of clearance upto 5 days.

Quantitative estimation of phosphate solubilisation: Bacterial cells were inoculated into the Pikovskaya's broth[17] after adjusting the O.D.600 to 0.8. Un-inoculated media served as blank. After incubation for 72 h, pH of the medium was recorded with a pH meter, cell numbers were estimated by standard plate count method and amount of inorganic phosphate released was measured by molybdenum-blue method[19].

Taxonomic identification of the isolates: Taxonomical identification of bacterial isolate YU-SS-C-67 was carried out through 16S rRNA gene sequencing. Genomic DNA was extracted from 48 h grown bacterial culture using genomic DNA extraction kit (MoBio Inc) as per manufacturer's instructions. Amplified 16S rRNA gene was sequenced using the BigDye terminator cycle sequencing kit and the nucleotide sequence was determined by an automatic DNA sequencer (ABI PRISM 310, Applied Biosystem, USA). Sequence data was aligned and compared with standard sequences in the GenBank using Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (NCBI). Further analysis of the sequences was performed using the software package MEGA (Molecular Evolutionary Genetic Analysis) version 5.0[20], after multiple alignment of data by Clustal_X[21]. Phylogenetic position of the isolate was then derived using a distance matrix method which includes clustering by neighbor joining and a discrete character based maximum parsimony method.

The 16S rRNA gene sequence of the isolate has been submitted to the GenBank database under the accession number KF514111.

Evaluation of U sensitivity of the isolate: Sensitivity of the bacterium to U toxicity was conducted by monitoring the cell viability as previously described[7]. A stock solution of U was prepared by dissolving uranyl nitrate hexahydrate in Milli-Q water and subsequently diluted to attain a concentration of 120 ppm U (VI). The isolate YU-SS-C-67 which was grown in Nutrient Broth overnight was harvested by centrifugation (5000 rpm, 20 min), washed and transferred to 0.1 N NaCl solution of pH 4 with or without U (120 ppm) and incubated at 32 ± 2 °C with continuous shaking (120 rpm). After 6 h of incubation, aliquots of solution were withdrawn, diluted with normal saline (0.85% NaCl) solution, plated on nutrient agar, incubated for 48 h and enumerated. *Escherichia coli* ATCC 25922^T was used as a reference strain.

Statistical analysis: All the values reported represent the mean of triplicates ($n = 3$) which were analyzed by one way analysis of variance (ANOVA) using the software package STATISTICA. Results were considered to be significantly different if $p < 0.01$.

Results and Discussion

Isolation and identification of the isolate: YU-SS-C-67 isolated from tube well water of Gogi is a gram positive bacterium producing yellow colony on Pikovskaya's agar. Taxonomic and phylogenetic analysis by 16S rRNA gene sequencing identified the isolate as *Curtobacterium* sp. YU-SS-C-67 showing maximum similarity to the type strain *Curtobacterium luteum* DSM 20542^T. Figure 1 shows the relationship between the strain YU-SS-C-67 with members of *Curtobacterium* and other related genera based on 16S rRNA gene sequences.

Determination of phosphate solubilization ability: Table 1 summarizes the SI index, cell number, phosphate released and change in media pH after 72 h incubation of the bacterium *Curtobacterium* sp. YU-SS-C-67. When grown on Pikovskaya's agar medium, the isolate showed clear zone around the colony indicating the solubilization of tri-calcium phosphate present in the media. On incubation in Pikovskaya's broth for 72 h, there was a drop in the media pH accompanied by the release of phosphate. Several studies have reported the role of organic acids like lactic, citric, malic, succinic, propionic acids in the solubilization of tri-calcium phosphate[11, 22]. The decrease in the pH of the media as observed in the present study can also be attributed to the production of organic acids by the isolate *Curtobacterium* sp. YU-SS-C-67.

Table-1
Variations in SI, cell density, ortho-phosphate and pH change observed after 72 h bacterial incubation in the Pikovskaya's broth

Isolates	SI	Log CFU/mL	Phosphate released (mgL^{-1})	pH of the culture broth
Control	-	-	41.19 ± 1.49	6.93 ± 0.1
Curtobacterium sp. YU-SS-C-67	1.51 ± 0.34	10.28 ± 0.64	271.13 ± 18.32	5.8 ± 0.03

Table-2
Number of viable cells as determined by log of Colony forming unit (CFU)

Isolates	[1]	[2]	[3]
Curtobacterium sp. YU-SS-C-67	12.12 ± 0.11	11.81 ± 0.17	10.25 ± 0.23
E.Coli	12.56 ± 0.44	12.07 ± 0.28	7.89 ± 0.03

Column [1]: log CFU/mL after washing the cells in pH 4 solution with 0.1 N NaCl on removal from the growth medium. Column [2]: log CFU/mL after incubation for 6 in pH 4 solution with 0.1 N NaCl and column [3]: log CFU/mL after incubation for 6 in pH 4 solution with 0.1 NaCl containing 120 ppm U (VI)

Uranium sensitivity study of the isolate: The results of U sensitivity study of the *Curtobacterium* sp. YU-SS-C-67 is shown in table 2. The difference between the colony forming unit (CFU) of columns 1 and 2 indicates the loss of viability due to nutrient deficiency and acidic condition. The difference among columns 1 and 3 indicates the combined effects of U toxicity, nutrient deficiency and acidic condition on cell viability. Comparison between columns 2 and 3 indicates loss of viability only due to U toxicity. The percent reduction in the cell number due to nutrient deprived acidic condition was 2.56% for *Curtobacterium* sp. YU-SS-C-67 as compared to 3.88% for *E. coli* which shows that both the isolates were able to survive in the nutrient deprived acidic condition. On incubation with 120 ppm U for 6 h, *Curtobacterium* sp. YU-SS-C-67 showed a minimum reduction in the cell number of 12.89% which is significantly lower ($p < 0.01$) in comparison to 33.22% reduction of cell number observed in the reference strain *E. coli* ATCC 25922^T. This shows that the *Curtobacterium* sp. YU-SS-C-67 is able to tolerate the chemical toxicity of U. Presence of diverse community of bacteria belonging to genus *Pantoea, Pseudomonas, Enterobacter and Bacillus* were previously reported in soil and sediments collected from U contaminated sites[23]. Also, some studies have investigated the tolerance of the bacteria to U and other heavy metals[24]. These bacteria with the potential to tolerate the metal toxicity, interact with metals promoting their removal, and therefore offer interesting opportunities for biotechnological applications involving treatment of contaminated sites[10, 24, 25].

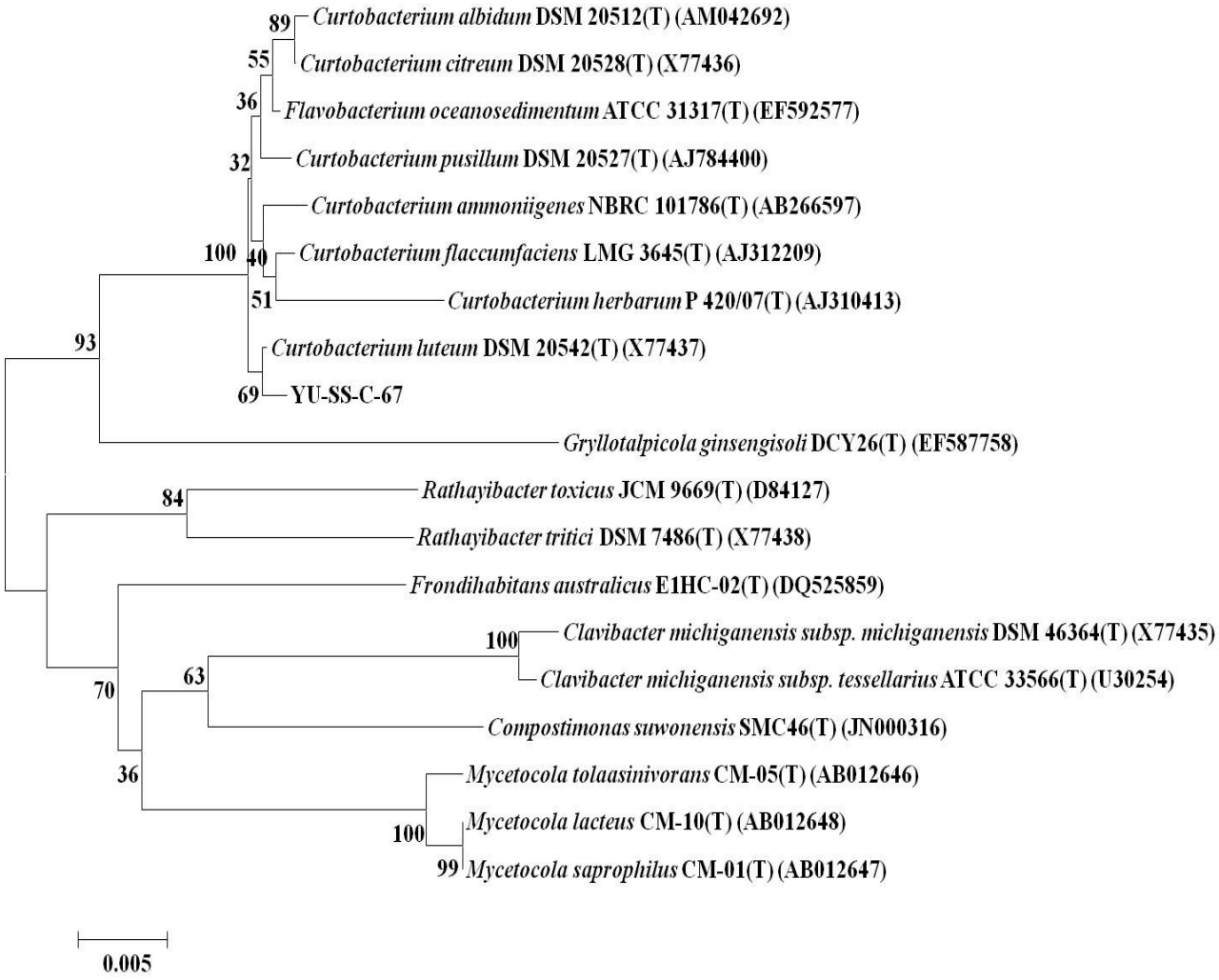

Figure-1
Phylogenetic analysis of the isolate YU-SS-C-67 based on 16S rRNA gene sequences. Distances and clustering were performed by using Neighbor-Joining method with the software package MEGA version 5. Bootstrap values based on 1,000 replications are listed as percentages at the branching points

Conclusion

With the ability to influence the geochemical processes and affect the mobility of the metal and contaminant, microorganisms show promise in the remediation process. The isolate *Curtobacterium* sp. YU-SS-C-67 with dual properties of phosphate solubilization and U tolerance can find application in the remediation of U via phosphate based mineralization, phytoremediation and phytorestoration of the contaminated sites. As the bacteria isolated from contaminated sites are known to present higher resistance to the prevailing toxic elements, further research should focus on the isolation of these indigenous bacteria for various bioremediation applications.

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