

Response of Hanford Site Soil *Arthrobacter* Isolates to Uranium Contamination

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ABSTRACT

Uranium is one of the most prevalent radiological groundwater and soil contaminants at Hanford, the U.S. Department of Energy (DOE) site, Washington State, USA. Bioremediation strategies, such as injections of a soluble sodium tripolyphosphate amendment into the contaminated groundwater in order to sequester uranium through the formation of insoluble uranyl phosphate minerals, may have resulted in providing a readily available nutrient for various microorganisms that thrive under oligotrophic conditions in the form of aqueous orthophosphate; this may lead to an increase in their growth. Increased microbial activity that influences meta-autunite stability is an important geochemical factor affecting the uranium dissolution and transport in the specific environmental conditions present at the Hanford Site's subsurface. However, the role of bacteria in phosphate remediation technology and the interactions between uranyl phosphates and the microbes are unknown. The lack of knowledge of the long-term stability of the sequestered uranium in the subsurface that may undergo subsequent remobilization severely limit the design of remediation strategies for uranium contaminated sites. *Arthrobacter* sp., a genus of gram-positive aerobic bacteria are known for their ability to survive for elongated periods under adverse environmental conditions, account for about 25% of the microbial population in Hanford soil. Five *Arthrobacter* strains, previously isolated from the Hanford subsurface, were obtained from the Subsurface Microbial Culture Collection (SMCC) (Florida State University). The interaction studies consisted of acclimating different microbial strains in various substrates; subsequent pre-screening tests using direct cell count and electrical cell substrate impedance sensing (ECIS) methods helped identify the most rapidly growing and uranium resistant strain of *Arthrobacter* sp. to be used in the following autunite mineral leaching experiments. The results obtained at these specific uranium concentrations, *Arthrobacter* sp. exhibit uranium resistance for bacterial cell growth in the various substrates have shown that G954 is the fastest growing strain while no significant difference in cell growth is observed between the G975 and G968 strains. The uranium tolerance assessment experiments have helped identify G975 as the most resistant *Arthrobacter* strain.

These experiments have indeed provided a first insight into the essential defining parameters, which are needed in support of the ensuing microbial meta-autunite dissolution experiments mimicking the oligotrophic Hanford environment.

INTRODUCTION

Uranium has been recognized as one of the most widespread groundwater contaminants at Hanford, the U.S. Department of Energy (DOE) site in Washington State [1]. Injections of a soluble sodium tripolyphosphate amendment into the uranium contaminated soil and groundwater have been shown to effectively sequester uranium through the formation of insoluble uranyl phosphate minerals [1, 2]. Polyphosphate undergoes hydrolysis in aqueous solutions to form orthophosphate, which serves as a readily available nutrient for the various microorganisms that thrive under the specific conditions, leading to an increase in their growth. Typically, in oligotrophic groundwater environments, inorganic nutrients such as phosphorus (P) are scarce and often decrease activity of the native microbial community. Miettinen et al., [3] found that microbial growth in water is highly regulated not only by organic carbon but also by the availability of phosphorus even in a very small concentration, often as low as 1µg. Other inorganic nutrients such as sodium (N), potassium (K), magnesium (Mg), and calcium (Ca) don't significantly affect the microbial growth. Thus, one could expect an increase in the microbial activity at the site following a polyphosphate injection into the groundwater since indigenous microorganisms might utilize available P from the polyphosphate amendments. The presence of rapidly adapting bacterial populations in sediment could strongly influence the migration/dissolution of uranium [4]. Other data reported in literature [5-8] suggests that indigenous microorganisms could enhance the mobility of heavy metals including the uranyl species [4]. However, there is limited information on the effect of microorganisms on the stability of uranyl phosphates and dissolution of uranium from mineral in the Hanford Site environmental conditions. Therefore, understanding the role of bacteria in phosphate remediation technology and the interactions between uranyl phosphates and the microbes are very important.

Arthrobacter sp., being rather immune to desiccation are found to thrive under very limited nutrients, and extreme environmental conditions that are present at the Hanford Site. Additionally, this microorganism was found to be the most prevalent genera underneath the leaking radionuclide storage tanks at the Hanford Site [9].

Metal-resistant bacteria, such as the *Arthrobacter* sp., are useful tools to accurately understand the microbiology of polluted environments as they serve as bioindicators for environmental monitoring and bio-remediation in polluted habitats [10]. *Arthrobacter* tolerance to uranium to survive in the presence of dissolved uranium (VI) is an essential step toward the understanding the microbial-uranium interactions.

The objective of the current study is to conduct prescreening tests with the *Arthrobacter* sp. isolates from Hanford soil to determine the bacterial cell density, and growth both in the absence and in presence of uranium and to identify the most uranium resistant strain, which is to be used in the subsequent autunite mineral leaching experiments.

MATERIALS AND METHODS

The current study was aimed towards the acclimation of the bacterial culture and in the process, determination of the most rapidly growing strain amongst the ones obtained from the Subsurface Microbial Culture Collection (SMCC). Studies such as direct count (by means of a hemocytometer), and Electrical Cell-Substrate Impedance Sensing (ECIS) were utilized for the same.

Microbial Culture

Five strains of *Arthrobacter* sp. were obtained from the Subsurface Microbial Culture Collection (SMCC), maintained at the Florida State University, Tallahassee, FL. The collection was established by DOE and contains nearly 10,000 strains of microorganisms (mostly bacteria) isolated from terrestrial subsurface environments.

The strains obtained for the present study were *Arthrobacter globiformis* (G929 and G954), and *Arthrobacter oxydans* (G968, G975, G977) based on the phylogenetic analysis of 16S rRNA gene sequences [11], out of which experimentation has been conducted with only G975, G968 and G954, due to contamination of the remaining strains by *Streptomyces*.

The strains were cultured in a complex media, PYTG, consisting of 5 g/L peptone, 5 g/L tryptone, 10 g/L yeast extract, 10 g/L glucose, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.07 g/L $CaCl_2 \cdot 2H_2O$ and 15 g/L agar. These studies were carried out in both 1% and 5 % liquid culture media and agar plates.

Members of the genus *Arthrobacter* are aerobic in nature; however, they have been observed to possess unexpected fermentation capabilities and a number of these bacteria can easily switch from aerobic to anaerobic respiration by rapid assimilation of glucose from the media [7, 10, 12]. In order to avoid the occurrence of any possible fermentation during bacterial growth, we have attempted to grow the *Arthrobacter* strains in the presence of non-fermentable low molecular weight substrates such as PYTF (Formate), PYTL (Lactate), and PYTA (Acetate). The cultures grown in PYTA were eliminated from further experiments due to an extremely low growth rate. With the final goal of these experiments being the study of uranium (autunite) leaching by the microbial species, the selection of appropriate substrates played a very important role. Bacterial cultures in low-molecular weight substrates exhibit a low affinity for uranyl species (VI) and have minimal effect on the leaching process.

The *Arthrobacter* strains were grown to reach confluence in 50 ml polypropylene sterile centrifuge tubes, with a foam stopper to simulate aerobic conditions in 10 ml of PYTG or PYTF or PYTL media at 29°C in the shaker/incubator. The cell density (cells/ml) was calculated with the help of a hemocytometer (Fisher Scientific, Pittsburg, PA). The hemocytometer consists of a thick glass slide with a rectangular indentation, creating a chamber that is engraved with a grid of perpendicular lines. Having known the area bounded by the lines as well as the depth of the chamber, the cell density in a specific volume of fluid, can be calculated and the concentration of cells in the media overall can be obtained.

Electrical Cell-Substrate Impedance Sensing (ECIS)

Studies, such as the Electrical Cell-Substrate Impedance Sensing (ECIS), conducted on bacterial culture acclimation represented the preliminary assessment of the bacterial growth. This technique was used for the preliminary monitoring of the cell proliferation and growth taken from the culture that was initiated in a flask.

ECIS is considered to be a novel method to perform real time cell monitoring without the utilization of radioactive tracers, or biological markers [13]. The alternating current (AC) impedance analysis, based on measuring the change in impedance of a small electrode to AC current flow, is used in the assessment. The heart of the measurement is a specialized cell culture array with eight individual wells for measurement. The base of the device consists of an array of gold film electrodes that connect the ECIS electronics to each of the wells. The measurements are used to monitor cell proliferation and to determine how various changes in cell and culture conditions affect the rates at which the cell monolayer approaches confluence. In order to

accomplish this, the arrays are inoculated with a low cell density providing room for the dividing cell population. As the cell number increases, the amount of electrode area covered with the spread cells grows accordingly, causing the electrode impedance to rise. The impedance change can be correlated to the relative cell proliferation rates or, more accurately, the rate at which the substrate becomes occupied with attached cells. The cell attachment data uses AC currents at a frequency of 4000 Hz.

The experiments were carried out using 8W1E PCB culture ware, (Applied Biophysics Inc., NY) consisting of eight polycarbonate/polystyrene squared wells equipped with a working electrode. The wells were injected with cells ($N_0=10^6$ cfu) dispersed in media (PYTG, PYTF or PYTL) in the exponential phase or with a control consisting of media alone to constitute a constant volume of 300 μ l. The temperature of the incubator was maintained at 29°C during the course of the experiments carried out with the ECIS device (24-72h). After the completion of the experiment, the cells (N_t) were counted and an accurate estimation of growth was obtained.

RESULTS AND DISCUSSION

Characterization of *Arthrobacter* sp.

Arthrobacter sp. is a genus of gram-positive aerobic bacteria, commonly found in soil [14] that thrive with very limited nutrients and under the extreme environmental conditions that are present at the Hanford Site. The most common isolates in the Hanford sediments have been the *Arthrobacter* sp. [9], which account for about 25% of the total microbial population.

This genus is distinctive because of its unusual cell division, wherein the outer bacterial cell wall ruptures at a joint; this type of cell division, in which rods break into cocci are referred to as 'reversion'. Under the microscope, these dividing cells appear 'V' shaped. Other notable characteristics are that it can use pyridone as its sole carbon source, and that its cocci are resistant to desiccation and starvation [12].

It was observed during the course of this study, that the species in this genus are gram-positive obligate aerobic bacteria that are rod shaped during the phase of exponential growth and cocci in their stationary phase. The morphology of G975, G954 and G968, as observed under a ML 2000 microscope (Meiji Techno Co. Ltd., Japan) with 100X magnification are shown in Fig. 1(a, b, c).

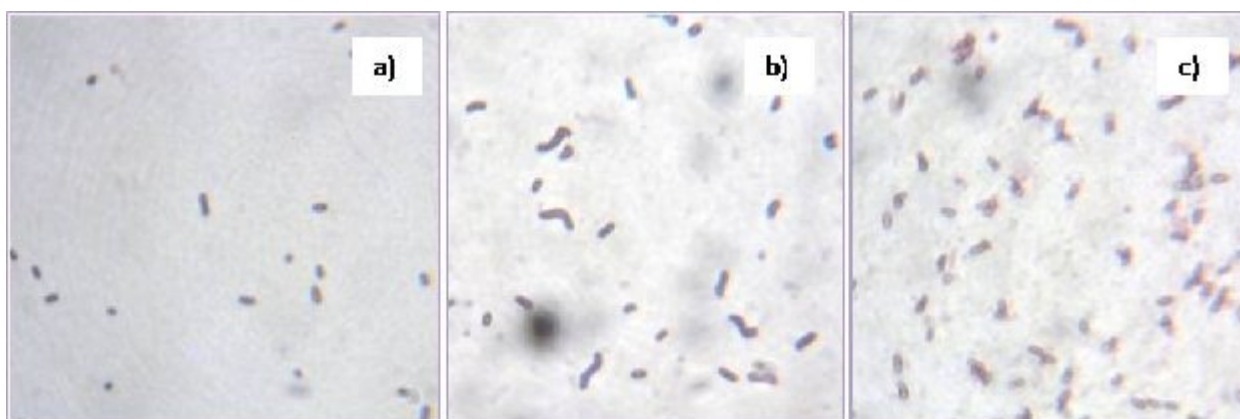


Fig. 1. Morphology of (a) G975 (b) G954 (c) G968

Cell Density Measurements

The cell density was obtained by direct cell count measurements with the hemocytometer at 20, 48 and 75 hours in PYTG as shown in Fig. 2 for all the working strains of *Arthrobacter*.

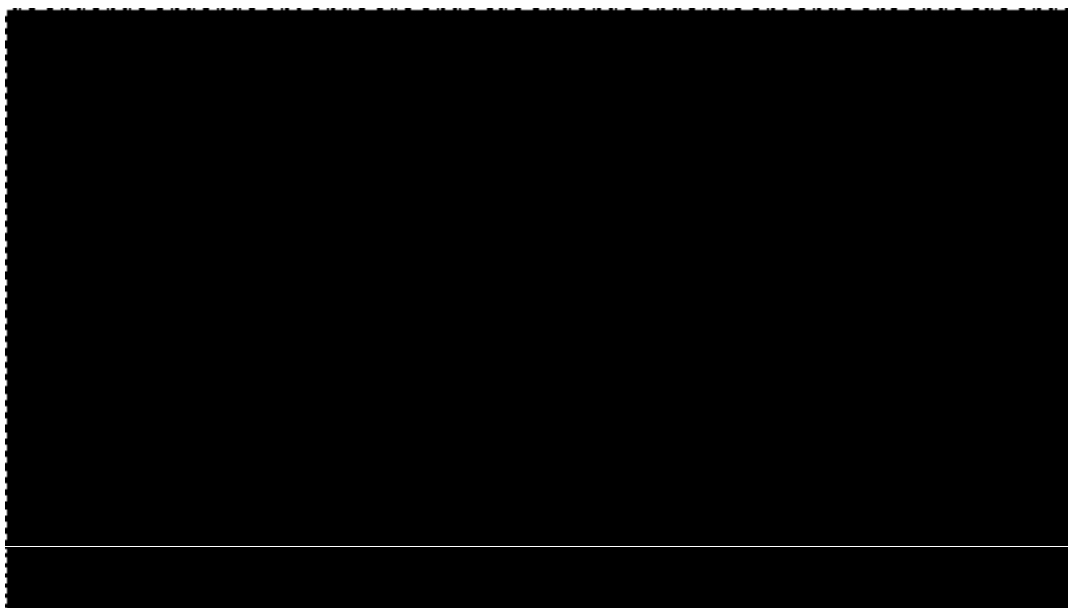


Fig. 2: Cell growth for *Arthrobacter* at specific time intervals in PYTG media

Additional cell density studies were performed with G975, G968 and G954 in PYTG, PYTL, and PYTF for a period of 72 h to make an accurate assessment of growth rate in these media as shown in Fig. 3. To ensure uniform growth to enable comparison amongst the various strains in different substrates, the initial cell concentration was maintained at 10^6 cfu. As seen in the figure, while minor differences in the growth can be observed at 6.5 h, at a period of 72 h, any difference in the bacterial cell growth is almost indistinguishable. However, this assessment doesn't allow us to perform a quantification of the growth rate for the *Arthrobacter* sp. under study. This will be obtained from the future ECIS studies.

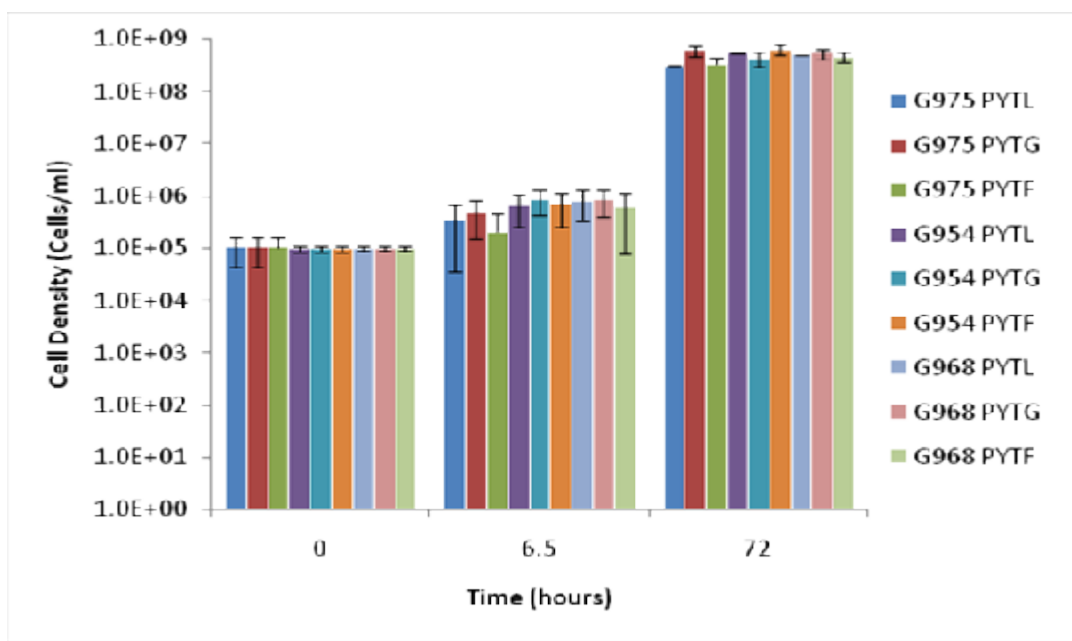


Fig. 3: Cell density measurements for *Arthrobacter* growing in PYTL, PYTG, and PYTF

ECIS

The wells in the ECIS microarray were injected with cells ($N_0 = 10^6$ cfu) in the exponential phase dispersed in media containing about 24 ppm of uranyl species (U(VI)); the control consisted of media along with U(VI) and the microbial cells dispersed in media without uranium to constitute a constant total volume of 300 μ l. The temperature of the incubator was maintained at 29°C during the course of the experiments carried out with the ECIS device (24-72h). After the completion of the experiment, the cells (N_t) were counted and an estimation of growth was obtained, as seen in Fig. 4.

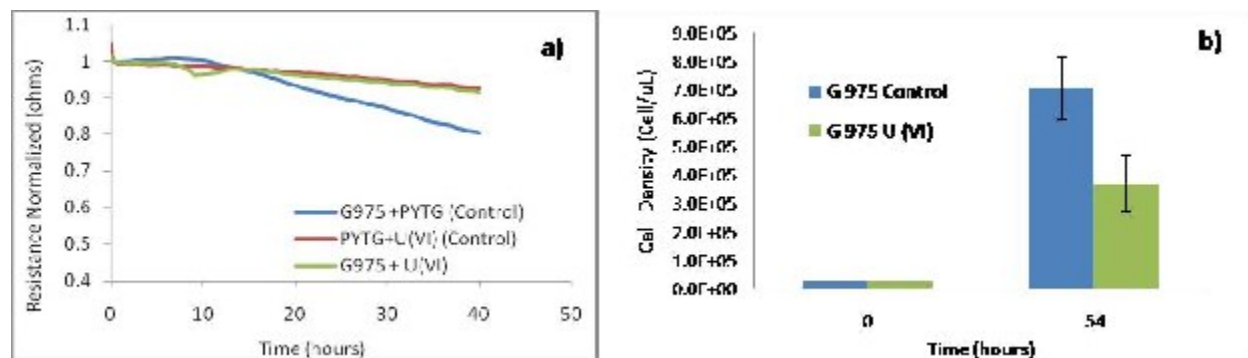


Fig. 4. a) G975 resistance to U(VI) using ECIS; b) Estimation of U tolerance based on cell count (N_0 , N_t)

The arrays of the ECIS instrument were inoculated with a low cell density such that it provided enough room for the dividing microbial cell population. As the cell number increased, the amount of electrode area covered with the spread cells grew accordingly, causing the electrode resistance to rise.

The initial rise in the curve, seen in Fig. 4(a) was due to the suspended cells (control consisting of G975) continuously settling down, attaching to the electrodes, and effectively blocking the area available for current. Then the cells began to develop focal adhesions, spread, and form a monolayer on the surface. Various small changes in the cell-electrode interaction due to cell motion caused the impedance to fluctuate with time. The change in the resistance (normalized to an initial value) could be correlated to the cell proliferation and attachment. However, after a period of 15 h, the cells reached senescence and as the nutrient in the media were consumed, a gradual decrease in the resistance was observed due to bacterial cell death.

In the case of the G975 inoculated in the media containing U(VI), it was observed that the cell growth was slower during the first 15 h and gradually increased as the cells acclimatized to the presence of U(VI).

Cells counts performed prior to and after the ECIS experimentation, helped us make an accurate assessment of uranium resistance behavior of *Arthrobacter* sp. as seen in Fig. 4(b).

As seen in the figure, the experiment was performed such that N_0 was maintained at a constant level; N_t (total cell count) provided a clear picture of microbial growth, both in the presence and absence of the uranyl species. While the cell density was observed to be higher in G975 in media without U(VI), it can be observed from Fig. 4(a) that the growth rate was higher in the cells from 20-40 h, once it developed resistance to the uranyl species.

No cell viability assay was performed, so N_t gave us an estimation only of the total cell count without distinguishing between alive and dead cells.

Future experiments need to be conducted in the presence of varying uranium concentration (0-50 ppm) for a period of 0-40 h to understand the mechanism of uranium resistance with the help of

ECIS. Experiments wherein, the specific concentrations of U(VI) are injected at specific time intervals (2, 3, 5 h) are to be carried out to elucidate the uranium tolerance mechanism of *Arthrobacter*.

Table I shows a summary of additional uranium resistance studies that have been conducted for *Arthrobacter* using 11.9, 23.8, 35.7 ppm of U(VI) at time intervals of 20.5 h, 28 h and 75 h (G968 only).

Table I: Cell Density Reduction (%) for G975, G968 and G954

Uranyl Nitrate Concentration	Bacterial Strain	Time, hr		
		20.5	48	75
11.9 ppm	G975	29.4±12	86.6±2.9	
	G968	71.0±7.9	93.7±1.8	93.2±7.8
	G954	87.7±3.5	94.1±1.1	
23.8 ppm	G975	47.0±10.3	83.1±3.6	
	G968	87.8±3.3	97.0±0.9	95.1±5.5
	G954	70.8±5.1	86.5±1.8	
35.7 ppm	G975	92.5±1.7	92.6±1.4	
	G968	95.0±1.3	98.3±0.6	96.0±0.2
	G954	93.0±0.9	98.0±0.6	

CONCLUSION

Interactions amongst radionuclides and microbes that promote their precipitation and immobilization are promising strategies for the treatment and cleanup of the contaminated subsurface [1, 4, 5, 8]. At sites where the concentrations of metal contaminants can reach toxic levels, such as the Hanford Site, the metal resistances of indigenous microbial populations serve as a critical parameter for the success of in situ bioremediation efforts.

The genus *Arthrobacter* are characterized by their unique ability to grow as two distinct cell shapes, forming either spherical or rod-shaped cells, depending upon the culture medium and growth phase, as seen from the characterization studies.

The results obtained for cell growth in the various substrates has shown us that G954 is observed to be the fastest growing strain. No significant difference in cell growth was observed between the G975 and G968 strains.

Assessment of the uranium resistance of *Arthrobacter* sp. has been carried out by culturing the bacterial cells in various concentrations of uranyl nitrate (11.9, 23.8, 35.7 ppm). The cell density data has shown that G975 is the most uranium tolerant strain followed by G968, and G954. However, as the values of the standard deviations among the G968 and G954 strains overlap, any difference between them is indistinguishable. We can demonstrate at these specific uranium concentrations, *Arthrobacter* sp. exhibit uranium resistance because the bacterial cell density increases with time despite a severe reduction when compared to its control at the same time point. Additionally, the ECIS experiments have confirmed the presence of uranium resistance of G975.

Having witnessed the resistance of the microorganisms under study to the toxic contaminants, the future experiments need to be designed over a larger uranium concentration range. Biosorption studies for *Arthrobacter* sp. need to be performed to elucidate the resistance

mechanism of the bacterial cells. Employing techniques such as Surface Plasmon Resonance (SPR) to study the microbial-uranium interaction in the future will ensure deeper understanding of the uranium tolerance mechanism.

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