Promoting Uranium Immobilization by the Activities of Microbial Phosphatases

Robert J. Martinez[1], Melanie J. Beazley[2], Samuel M. Webb[3], Martial Taillefert[2] (co-PI) and Patricia A. Sobecky[1] (PI)

[1]School of Biology, Georgia Institute of Technology, Atlanta Georgia

[2]School of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta Georgia

[3]Stanford Synchrotron Radiation Laboratory, Menlo Park, California

Abstract

The overall objective of this project is to examine the activity of nonspecific phosphohydrolases present in naturally occurring subsurface microorganisms for the purpose of promoting the immobilization of radionuclides through the production of uranium [U(VI)] phosphate precipitates. Specifically, we hypothesize that the precipitation of U(VI) phosphate minerals may be promoted through the microbial release and/or accumulation of PO_4^{3-} as a means to detoxify radionuclides and heavy metals. An experimental approach was designed to determine the extent of phosphatase activity in bacteria previously isolated from contaminated subsurface soils collected at the ERSP Field Research Center (FRC) in Oak Ridge, TN. Screening of 135 metal resistant isolates for phosphatase activity indicated the majority (75 of 135) exhibited a phosphatase-positive phenotype. During this phase of the project, a PCR based approach has also been designed to assay FRC isolates for the presence of one or more classes of the characterized non-specific acid phophastase (NSAP) genes likely to be involved in promoting U(VI) precipitation. Testing of a subset of Pb resistant (Pbr) *Arthrobacter*, *Bacillus* and *Rahnella* strains indicated 4 of the 9 Pbr isolates exhibited phosphatase phenotypes suggestive of the ability to bioprecipitate U(VI). Two FRC strains, a *Rahnella* sp. strain Y9602 and a *Bacillus* sp. strain Y9-2, were further characterized. The *Rahnella* sp. exhibited enhanced phosphatase activity relative to the *Bacillus* sp. Whole-cell enzyme assays identified a pH optimum of 5.5, and inorganic phosphate accumulated in pH 5.5 synthetic groundwater (designed to mimic FRC conditions) incubations of both strains in the presence of a model organophosphorus substrate provided as the sole C and P source. Kinetic experiments showed that these two organisms can grow in the presence of 200 μM dissolved uranium and that *Rahnella* is much more efficient in precipitating U(VI) than *Bacillus* sp. The precipitation of U(VI) must be mediated by biological activity as less than 3% soluble U(VI) was removed either from the abiotic or the heat-killed cell controls. Interestingly, the pH has a strong effect on growth and U(VI) biomineralization rates by *Rahnella*. Thermodynamic modeling identifies autunite-type minerals [$Ca(UO_2)_2(PO_4)_2$] as the precipitate likely formed in the synthetic FRC groundwater conditions at all pH investigated. Extended X-ray absorption fine structure measurements have recently confirmed that the precipitate found in these incubations is an autunite and meta-autunite-type mineral. A kinetic model of U biomineralization at the different pH indicates that hydrolysis of organophosphate can be described using simple Monod kinetics and that uranium precipitation is accelerated when monohydrogen phosphate is the main orthophosphate species in solution. Overall, these experiments and ongoing soil slurry incubations demonstrate that the biomineralization of U(VI) through the activity of phosphatase enzymes can be expressed in a wide range of geochemical conditions pertaining to the FRC site.

Image from http://public.ornl.gov/nabirfrc/photos_history.cfm

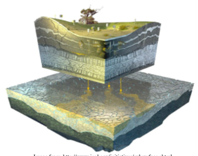


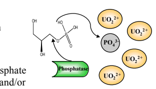
Image from: http://www.inel.gov/initiatives/subsurface.shtml

Hypotheses to be tested:

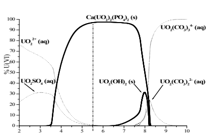
(1). Non-specific phosphophydrolases (acid phosphatases) provide subsurface microorganisms with resistance to heavy metals and lateral gene transfer has promoted the dissemination of this phosphatase-mediated resistance.

(2). Phosphatase activities of the subsurface bacterial populations can promote the immobilization of radionuclides via the formation of insoluble metal phosphate precipitates.

(3). Subsurface geochemical parameters (pH, nitrate) will affect phosphate mineral formation by altering microbial phosphatase activity and/or affecting the stability of the metal phosphate precipitates.

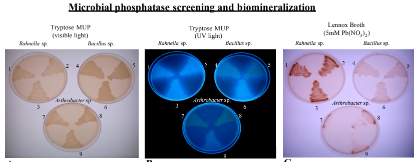


U(VI) – Phosphate Speciation as a Function of pH



•U(VI)-phosphate speciation at equilibrium predicted by MINEQL+ as a function of pH in simulated groundwater with UO_2^{2+} = 200 μM, ΣPO_4^{3-} = 200 μM , and $P_{CO_2} = 10^{-3.5}$.

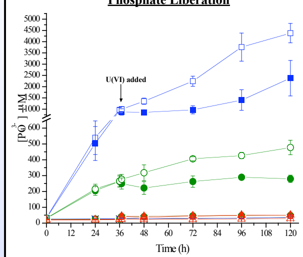
Microbial phosphatase screening and biomineralization



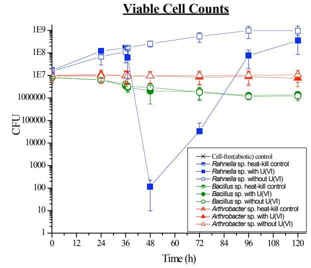
Tryptose MUP (4-methylumbelliferyl phosphate) agar plates and Tryptose Phosphate Methyl Green (TPMG) agar plates (not shown) were used to screen FRC isolates for phosphatase phenotypes **(A and B)**. 4-methylumbelliferyl phosphate (MUP) fluorescence as well as methyl green precipitation (not shown) indicated phosphatase positive phenotypes **(B)**. Lead (II) precipitation was also screened for isolates with and without phosphatase phenotype **(C)**. Insoluble lead (II) phosphate (brown precipitate) may result from extracellular phosphatase activity and/or potentially from depolymerization of cytoplasmic polyphosphate granules.

Uranium Biomineralization at pH 5.5

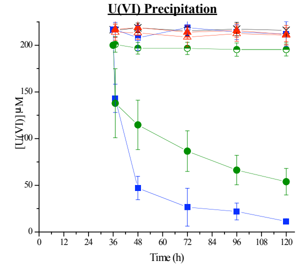
Phosphate Liberation



Viable Cell Counts

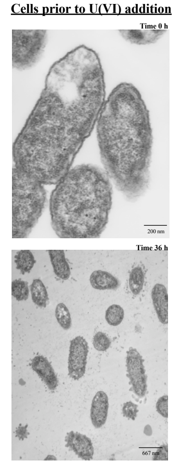


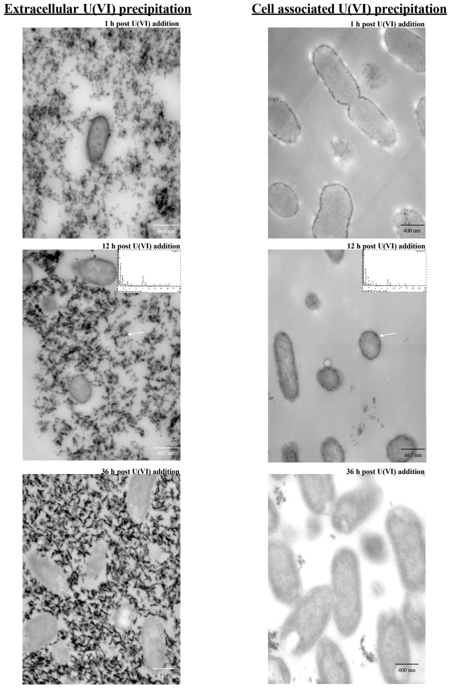
U(VI) Precipitation



Electron Microscopy of FRC *Rahnella* sp. Exposed to U(VI)

Cells prior to U(VI) addition | **Extracellular U(VI) precipitation** | **Cell associated U(VI) precipitation**

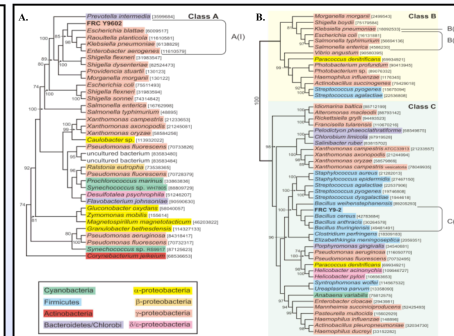


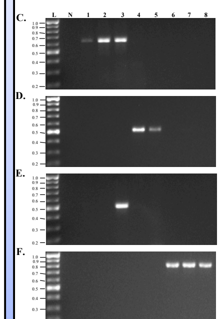


•Electron microscopy of FRC *Rahnella* sp. revealed membrane blebbing during incubation in simulated groundwater both prior to U(VI) addition and post U(VI) addition.

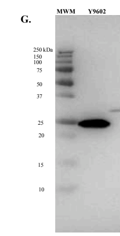
•Extracellular as well as cell associated electron-dense precipitates were shown via EDX analysis (regions marked by arrows) to be composed of uranium and phosphorus.

•Imaging of cells 36 h post U(VI) addition revealed a low distribution of cell associated U(VI) precipitates.



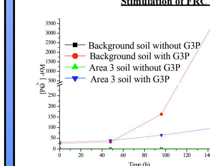


L - 1kb DNA ladder
N - No DNA template control
1 - FRC Y9602
2 - *Enterobacter aerogenes* (Class A control)
3 - *Klebsiella pneumoniae* (Class A and B control)
4 - *Escherichia coli* MG1655 (Class B control)
5 - *Salmonella enterica* (Class B control)
6 - *Bacillus cereus* (Class C control)
7 - *Bacillus thuringiensis* (Class C control)
8 - FRC Y9-2



Neighbor-joining analysis of acid phosphatase genes. Sequences were obtained by the aid of TASSER-Lite and the Enzyme Function Inference by Combined Approach (EFICAz) algorithm yielding Class A and Class B/Class C phylogenies, respectively **(A)** and **(B)**. Alignments generated for each class of acid phosphatase proteins were used to generate phylum-specific and species-specific PCR primers. Type-strains were used to determine primer specificity for Class A **(C)**, Class B **(D),** Class B (Kp) **(E),** and Class C **(F)**. Renatured whole cell lysates (100 μg/lane total protein) assayed for phosphatase activity in 100 mM Na acetate buffer at pH 5.5 with 5 mM phenolphthalein diphosphate substrate and 0.05 mg/ml methyl green **(G)**.

Stimulation of FRC Microbial Community Phosphatase Activity



•Stimulation of FRC soils with 10 mM G3P reveals phosphatase activity in uncontaminated and Area 3 (contaminated) soils.

•G3P hydrolysis in U(VI) contaminated soils reveals lower phosphate accumulation relative to uncontaminated soils.

•Future studies will examine the differences between microbial community structure and phosphatase activity between control and contaminated soils. In addition, the extent to which contaminated soils remove liberated phosphate by adsorption and promote U(VI) precipitation will be investigated.

Conclusions

• Biomineralization may be complementary to bioreduction in the immobilization of uranium from contaminated sites. The objective of this study was to determine if the phosphatase activity of subsurface microbes results in the release of sufficient PO_4^{3-} to complex and precipitate UO_2^{2+}.

• For this study, the gram-negative *Rahnella* sp. Y9-602 and *Bacillus* sp. Y9-2 isolated from the FRC were studied for their phosphatase activity in the presence of U(VI).

• Kinetic studies were conducted in solutions containing the *Rahnella* sp. and *Bacillus* sp. isolates and the organo-phosphate compound G3P to determine if phosphatase activity would promote the precipitation of uranium.

•Both *Rahnella* sp. and *Bacillus* sp. hydrolyzed sufficient phosphate to precipitate ~95% and ~ 73%, respectively, of the initial uranium in biogenic incubations.

•Electron microscopy and EDX analysis reveal extracellular and cell associated U(VI) phosphate precipitates.

•Preliminary evidence of FRC soil phosphatase activity reveals the potential for stimulating sufficient phosphate release to allow for U(VI) phosphate mineral formation.

Acknowledgements

This research is supported by the Office of Science (BER), U.S. Department of Energy, Grant No. DE-FG02-04ER63906. We thank Dave Watson (ORNL) for FRC samples, Dr. Adrian K. Arakaki (Georgia Tech) for phylogenetic computational analysis of NSAP genes, Hong Yi (Emory University) for electron microscopy imaging, Dr. Joan Hudson (Clemson University) for electron microscopy imaging and EDX analysis, David Giannantonio (Georgia Tech) and Alex Alverson (Georgia Tech) for technical assistance.