Promoting Uranium Immobilization by the Activities of Microbial Phosphatases

Robert J. Martinez[1], Melanie J. Beazley[2], Jarad J. Wilson[1], Martial Taillefert[2] and Patricia A. Sobecky[1]

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

Abstract

The overall goal of this project is to examine the role 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-}. During this phase of the project we have been conducting assays to determine the effects of pH, inorganic anions and organic ligands on U(VI) mineral formation and precipitation when FRC bacterial isolates were grown in simulated groundwater medium. The molecular characterization of FRC isolates has also been undertaken during this phase of the project. Analysis of a subset of gram-positive FRC isolates cultured from FRC soils (Areas 1, 2 and 3) and background sediments have indicated a higher percentage of isolates exhibiting phosphatase phenotypes (i.e., in particular those surmised to be PO_4^{3-}-irrepressible) relative to isolates from the reference site. A high percentage of strains that exhibited such putatively PO_4^{3-}-irrepressible phosphatase phenotypes were also resistant to the heavy metals lead and cadmium. Previous work on FRC strains, including *Arthrobacter*, *Bacillus* and *Rahnella* spp., has demonstrated differences in tolerance to U(VI) toxicity (200 µM) in the absence of organophosphate substrates. For example, *Arthrobacter* spp. exhibited the greatest tolerance to U(VI) while the *Rahnella* spp. have been shown to facilitate the precipitation of U(VI) from solution and the *Bacillus* spp. demonstrate the greatest sensitivity to acidic conditions and high concentrations of U(VI). PCR-based detection of FRC strains are being conducted to determine if non-specific acid phosphatases of the known molecular classes [i.e., classes A, B and C] are present in these FRC isolates. Additionally, these amplified phosphatases are being analyzed to determine whether or not there is evidence for the horizontal transfer of such genes amongst subsurface microbial populations. Microbially precipitated U(VI) phosphate minerals will be further analyzed via capillary electrophoresis and extended x-ray absorption fine structure spectroscopy to determine uranium speciation.

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

Hypotheses to be tested:

(1). Non-specific phosphohydrolases (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.

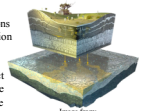
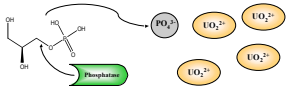


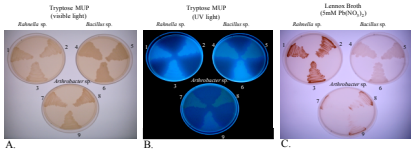
Image from: http://www.inel.gov/initiatives/subsurface.shtml

Biomineralization of soluble U(VI) through the activity of microbial phosphatases



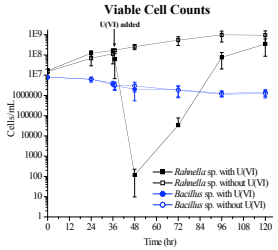
$$2PO_4^{3-}{}_{(aq)} + 3UO_2^{2+}{}_{(aq)} \rightarrow (UO_2)_3(PO_4)_{2(s)}$$

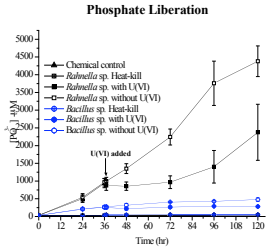
Microbial phosphatase screening and biomineralization

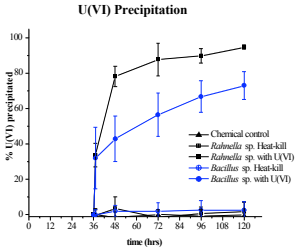


Tryptose MUP (4-methylumbelliferyl phosphate) agar plates and Tryptose Phosphate Methyl Green (TPMG) agar plates (not shown) were used to screen FRC isolates for phosphatase phenotypes (**A and B**). 4-methylumbelliferyl phosphate (MUP) fluorescence as well as methyl green precipitation (not shown) indicated 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 Challenge Assay at pH 5.5



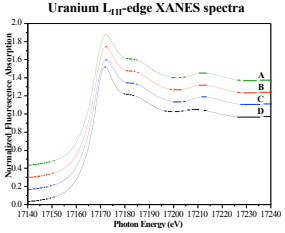




- The FRC *Rahnella* sp. demonstrated growth in simulated groundwater in the absence of U(VI). U(VI) significantly reduced viable cell counts for 12hr with a return to 10^8 cells/mL at T_{120}.
- Viable cell counts of the FRC *Bacillus* sp. decreased in both the presence or absence of U(VI).
- Both *Bacillus* sp. and *Rahnella* sp. demonstrated phosphatase activity in the presence of U(VI), with *Rahnella* sp. hydrolyzing significantly more G3P than *Bacillus* sp.
- With the addition of U(VI) at time 36 hour phosphate production did not begin to increase again for ~ 48 hours in *Rahnella* sp. incubations.
- In the absence of microbes, no significant chemical hydrolysis of G3P was observed.
- ~ 95% of initial U(VI) precipitated in assays with *Rahnella* sp. at pH 5.5 and is removed more quickly compared to *Bacillus* sp. incubations due to the increased concentration of phosphate in solution.
- ~ 73% of initial U(VI) precipitated in assays with *Bacillus* sp. at pH 5.5.
- Incubations with heat-killed cells indicate no U(VI) removed on cell membrane surfaces.

X-ray Absorption Spectroscopy Measurements

Uranium L_{III}-edge XANES spectra



Parameters Derived from Fitting of U L-edge EXAFS

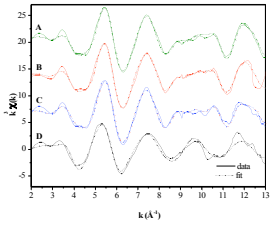
Sample		Oax	Oeq	P	POU MS[1]	OPOU MS[1]	PO dist[2]	Rfactor
A	CN	2	2.7 (0.4)	1.2 (0.4)	2.4	1.2	1.57 (0.06)	0.009
	R (Å)	1.79 (0.005)	2.28 (0.007)	3.59 (0.02)	3.72	3.84		
	σ^2 (Å^2)	0.003 (0.0003)	0.003 (0.0009)	0.003	0.003	0.003		
B	CN	2	3.1 (0.7)	0.7 (1.3)	1.5	0.7	1.4 (0.4)	0.0207
	R (Å)	1.79 (0.01)	2.28 (0.01)	3.54 (0.06)	3.62	3.7		
	σ^2 (Å^2)	0.0036 (0.0005)	0.004 (0.002)	0.003	0.003	0.003		
C	CN	2	3.7 (0.7)	1.6 (0.9)	3.1	1.6	1.56 (0.07)	0.0174
	R (Å)	1.79 (0.009)	2.27 (0.01)	3.58 (0.03)	3.71	3.84		
	σ^2 (Å^2)	0.0047 (0.0006)	0.005 (0.002)	0.003	0.003	0.003		
D	CN	2	1.6 (1)	1.7 (1)				0.0497
	R (Å)	1.81 (0.01)	2.31 (0.04)	2.47 (0.04)				
	σ^2 (Å^2)	0.0038 (0.0006)	0.005 (0.005)	0.005				

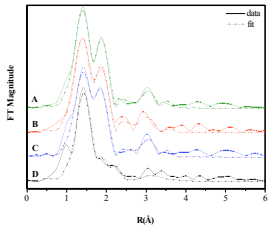
Errors are given in parentheses (no error means the value was fixed, or calculated from other parameters)
[1]MS denotes multiple scattering paths
[2]PO dist is the distance between the P-O in phosphate coordination (used for the MS paths)

Sample A = uranium phosphate precipitate from biological incubation of *Rahnella* sp. at pH 5.5.
Sample B = uranium phosphate/uranium hydroxide precipitate from biological incubation of *Rahnella* sp. at pH 7.0.
Sample C = chemical precipitation of uranium phosphate with 200 µM UO_2^{2+} and 200 µM NaH_2PO_4 at pH 5.5.
Sample D = chemical precipitation of uranium hydroxide at pH 7.0.

Note: all samples incubated in simulated groundwater and 200 µM UO_2^{2+}, with Samples A, B and D in the presence of 10 mM G3P.

Uranium L_{III}-edge EXAFS spectra



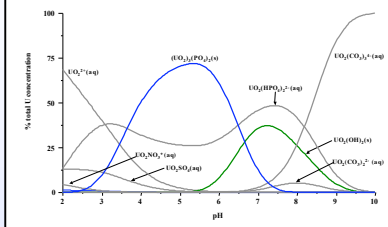


- *Rahnella* sp. (Samples A & B) in the presence of 10 mM G3P and simulated groundwater at pH 5.5 and pH 7.0, hydrolyzed sufficient PO_4^{3-} to precipitate 95 – 100% of uranium as uranium phosphate at pH 5.5 and a combination of uranium phosphate and uranium hydroxide at pH 7.0, as shown by EXAFS measurements on bulk biogenic samples.
- Uranium in all samples precipitated as U(VI) as indicated by both XANES and EXAFS.
- At pH 5.5, Samples A and C had approximately the same EXAFS structure, suggesting little difference between uranium mineralized in the presence of PO_4^{3-} from the microbially hydrolyzed organo-phosphate substrate and free inorganic orthophosphate.
- The U-P bond distance of ~3.6 Å indicated the uranyl ion formed a monodentate complex with phosphate in Samples A – C.
- Characteristics in the spectra between 8.5 and 10.5 Å^{-1} in Samples A – C was comparable to calcium autunite – $Ca(UO_2)_2(PO_4)_{2(s)}$.

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 for FRC samples, David Giannantonio and Alex Alverson for technical assistance. We thank Sam M. Webb for EXAFS analysis carried out at the Stanford Synchrotron Radiation Laboratory (SSRL), a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences.

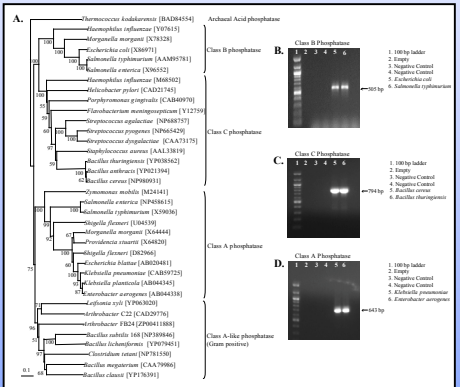
U(VI) – Phosphate Speciation as a Function of pH



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 $P_{CO_2} = 10^{-3.5}$

- At low pH uranium is mainly in the form of uranyl ions.
- As pH increases between 4 and 6 highly insoluble uranium phosphate compounds form
- Solid uranium hydroxide mineral forms at pH > 6
- Highly soluble uranium carbonate species form at pH > 8 and do not affect uranium phosphate precipitation

•Simulated groundwater consists of: 2 µM Fe^{3+}, 5 µM Mn^{4+}, 8 µM MoO_4^{2-}, 0.81 mM Mg^{2+}, 7.5 mM Na^+, 0.81 mM SO_4^{2-}, 0.41 mM Cl^-, 7.9 mM K^+, 0.20 mM Ca^{2+} and 15.4 mM NO_3^-



Neighbor-joining analysis of putative and functional acid phosphatases derived from completed genomes (**A**). Phlyogenetic relationship among the 3 characterized acid phosphatase groups is depicted in the rooted tree. Scale bar represent 0.1 changes per amino acid position. Alignments generated for each class of acid phosphatase proteins were used to generate phylum-specific PCR primers. Type-strains were used to determine primer specificity (**B**), (**C**) and (**D**). Phylum-specific primers will be used to determine lateral gene transfer of acid phosphatases in FRC cultured isolates.

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.
- Preliminary incubations indicated that pH and phosphate concentration were controlling factors for uranium phosphate precipitation. At pH<5.5, *Rahnella* sp. hydrolyzes less phosphate and uranium precipitation is minimal. At pH 7.0, Rahnella sp. hydrolyzes mM quantities of phosphate, however, uranium hydroxide forms. Our studies indicated that pH 5.5 is the optimal pH for both acid phosphatase activity and uranium phosphate precipitation.
- Both *Rahnella* sp. and *Bacillus* sp. hydrolyzed sufficient phosphate to precipitate ~95% and ~ 73%, respectively, of the initial uranium in biogenic incubations.
- X-ray absorption spectrometry indicates the formation of a uranium mineral similar to calcium autunite – $Ca(UO_2)_2(PO_4)_{2(s)}$.
- The hydrolysis of G3P by aerobically mediated microbial activity is driving the precipitation of U(VI). In addition, as thermodynamically predicted, our studies demonstrate that pH is a determining parameter for uranium phosphate precipitation.