Promoting Uranium Immobilization by the Activities of Microbial Phosphatases

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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 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.

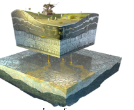
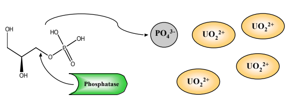
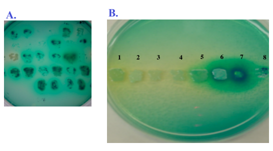


Image from: http://www.inel.gov/initiatives/subsurface.shtml

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



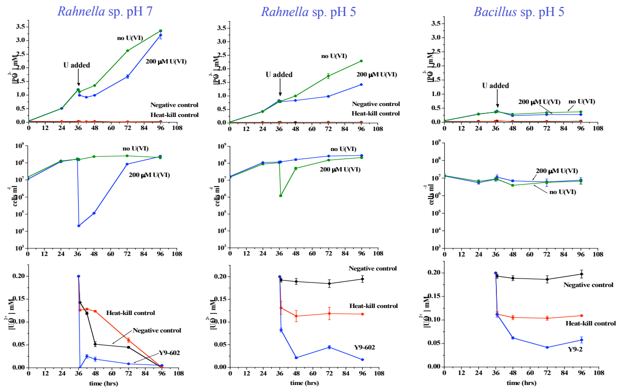
$$2PO_4^{3-}{}_{(aq)} + 3UO_2^{2+}{}_{(aq)} \rightarrow (UO_2)_3(PO_4)_{2(s)}$$

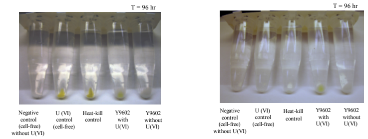


1. *P. veronii* (negative control)
2. *Arthrobacter* sp. strain AA20
3. *Arthrobacter* sp. strain V45
4. *Arthrobacter* sp. strain X34
5. *P. veronii* pJH123 (positive control)
6. *Rahnella* sp. strain Y9602
7. Unidentified strain AG15
8. *Bacillus* sp. strain X18

Figure 1. (A) Tryptose Phosphate Methyl Green (TPMG) agar plates used to screen FRC isolates for phosphatase phenotypes. **(B)** Phosphatase positive phenotypes appear as dark green colonies and/or cause the surrounding medium to darken. Negative phenotypes appear unstained. *Pseudomonas veronii* (previously shown not to liberate phosphate in growth assays) was used as a negative control and *Pseudomonas veronii* pJH123 (constitutively expressing the *phoA* gene) was used as a positive control

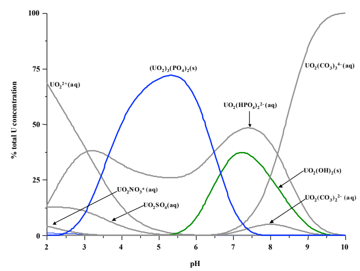
Incubation Results





- **Incubation parameters:**
  - **[U] = 200 μM**
  - **G3P = 10 mM**
  - **simulated groundwater**
- **Both bacterial species produce PO_4^{3-} in the presence of U(VI).**
- ***Rahnella* sp. produces 38% more PO_4^{3-} at pH 7 compared to pH 5.**
- **At pH 5 *Rahnella* sp. produces 148% more PO_4^{3-} than *Bacillus* sp.**
- ***Rahnella* sp. cell counts decrease after U(VI) addition, but rebound after 36-48 hours. Cells counts decrease more significantly at pH 7.**
- ***Bacillus* sp. cell counts are not affected by U addition.**
- **U(VI) is removed completely both abiotically and biotically at pH 7, suggesting precipitation of uranium phosphate and uranium hydroxide as evidenced by yellow precipitate in both control and biotic samples.**
- **U(VI) does not abiotically precipitate at pH 5.**
- **At pH 5 U(VI) biotically precipitates as evidenced by yellow precipitate in biotic sample.**
- **~40% of U(VI) is sorbed onto both *Rahnella* sp. and *Bacillus* sp. heat-killed cells.**
- **~50% and 30% of total U(VI) precipitates as uranium phosphate in *Rahnella* and *Bacillus* spp. incubations, respectively.**

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: 2.02 μM Fe^{3+}, 5.05 μM Mn^{4+}, 8.03 μM MoO_4^{2-}, 0.81 mM Mg^{2+}, 7.52 mM Na^+, 0.81 mM SO_4^{2-}, 0.41 mM Cl^-, 7.90 mM K^+, 0.20 mM Ca^{2+} and 15.4 mM NO_3^-

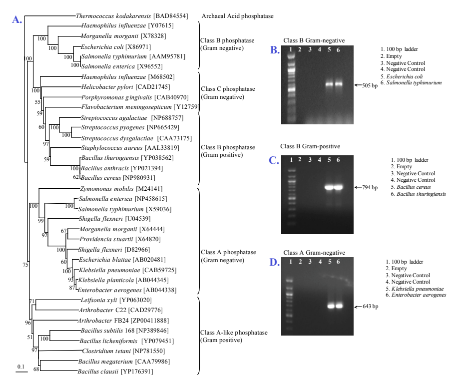


Figure 2. (A) Neighbor-joining analysis of putative and functional acid phosphatases derived from completed genomes. Phlyogenetic relationship among the 3 characterized acid phosphatase groups is depicted in the rooted tree. Scale bar represent 0.1 changes per amino acid position. Alignments generated 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.

Acknowledgements

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Experimental Design of Uranium Challenge Assay

- Incubations with *Bacillus* sp. (Y9-2) and *Rahnella* sp. (Y9-602) isolated from DOE Field Research Center (FRC), Oak Ridge, TN (Area 3)
- Lab incubation conditions:
  - Starting innoculum consisted of 10^7 cells/ml of FRC *Bacillus* or *Rahnella* spp.
  - simulated groundwater media with 15.4 mM nitrate as N-source
  - 10 mM glycerol-3-phosphate (G3P)
  - pH 5, pH 7
  - 30°C, 200 rpm
  - 200 μM uranium (from uranyl acetate stock; Spectrum)
- Uranium analysis by ICP-MS (Agilent 7500a Series)
- ΣPO_4^{3-} analysis by spectrophotometry (Murphy and Riley, 1962)
- Viable cell counts performed to determine cell growth.

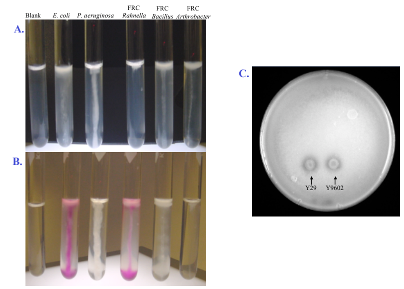

Figure 3. FRC *Arthrobacter* sp. strain X34, *Bacillus* sp. strain Y9-2 and *Rahnella* sp. strain Y9602 were assayed for physiological processes such as nitrate respiration **(A)** and **(B)** and solubilization of hydroxylapatite **(C)**.

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+}
- To date, the majority of the lead-resistant, phosphatase positive isolates obtained from the FRC contaminated soils are Gram-positive *Bacillus* and *Arthrobacter* species. Enrichment cultures, using phytic acid and other phosphatase substrates, will be developed to obtain additional Gram-positive and Gram-negative isolates.
- 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.
- Sufficient PO_4^{3-} is released from an organo-phosphate substrate to precipitate presumably as a uranium phosphate mineral.
- 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.

Future Directions

- Molecular characterizations of non-specific acid phosphatases from select FRC strains, specifically, determining substrate range, enzyme activity, optimal pH and identification of laterally transferred phosphatases using PCR primer sets developed in this project.
- Determine localization of phosphatases (periplasmic, surface, exocellullar) via X-ray microscopy.
- The results of this study provide positive indications that the removal of U(VI) from contaminated systems may be significantly enhanced through microbial enzymatic processes. Further study is needed to determine the speciation of the U(VI) complexes and the solid phase formed via microbially precipitated U(VI) phosphate minerals will be further analyzed by capillary electrophoresis and extended x-ray absorption fine structure spectroscopy