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Title: Molecular mechanisms and kinetics of microbial anaerobic nitrate-dependent U(IV) and Fe(II) oxidation
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Abstract
In this project, we combined molecular genetic, spectroscopic, and microscopic techniques with kinetic and reactive transport studies to describe and quantify biotic and abiotic mechanisms underlying anaerobic, nitrate-dependent U(IV) and Fe(II) oxidation, which influences the long-term efficacy of *in situ* reductive immobilization of uranium at DOE sites. In these studies, *Thiobacillus denitrificans*, an autotrophic bacterium that catalyzes anaerobic U(IV) and Fe(II) oxidation, was used to examine coupled oxidation-reduction processes under either biotic (enzymatic) or abiotic conditions in batch and column experiments with biogenically produced $U^{IV}O_2(s)$. Synthesis and quantitative analysis of coupled chemical and transport processes were done with the reactive transport modeling code Crunchflow. Research focused on identifying the primary redox proteins that catalyze metal oxidation, environmental factors that influence protein expression, and molecular-scale geochemical factors that control the rates of biotic and abiotic oxidation.

Results
In studies of the anaerobic, oxidative dissolution of biogenically produced U(IV)-oxide, either chemical oxidants (nitrate or nitrite) or enzymatic catalysis by *Thiobacillus denitrificans*, a chemolithoautotrophic bacterium capable of nitrate-dependent U(IV) oxidation, were compared in flow-through column experiments. Biogenic $UO_2(s)$ was synthesized under anaerobic conditions (using *S. oneidensis* strain MR-1) following previously published methods. Mixtures of biogenic $UO_2(s)$ and quartz were packed into 1 mL or 5 mL polypropylene columns. For experiments in the presence of *T. denitrificans,* cells were harvested anaerobically, washed and resuspended, mixed with quartz and biogenic $UO_2(s)$, and packed into the columns. Oxidation experiments were carried out in the dark at 25±2°C in an anaerobic glove box (10% H_2, 90% N_2) in buffered solution (MOPS, pH ~ 7.2). Total U release from columns was measured by ICP-MS. Changes in $UO_2(s)$ speciation after reaction were examined by synchrotron X-ray absorption spectroscopy (XAS) in bulk samples retrieved from column experiments compared with unreacted $UO_2(s)$. Uranium release behavior under different experimental conditions was analyzed and quantified using a reactive transport model that included thermodynamic solubility, irreversible overall abiotic and biotic kinetic reactions, and uranyl sorption.

Abiotic oxidation of $UO_2(s)$ in the presence of nitrate (1–20 mM) under anaerobic conditions is slow but faster than control experiments of non-oxidative dissolution (with NaCl solutions). Non-oxidative, abiotic rates in column experiments modeled with Crunchflow were similar to published rates in prior experiments using thin-film or continuous stirred-flow reactors. Abiotic

$UO_2(s)$ oxidation by nitrite (0.1–20 mM) is faster than non-oxidative dissolution by about 1-2 orders of magnitude. In the presence of *T. denitrificans* and dissolved nitrate (1-10 mM), higher rates of dissolved U release were observed compared with abiotic controls, suggesting that *T. denitrificans* catalyzed the oxidative dissolution of $UO_2(s)$ in addition to abiotic oxidation pathways, although there was variability among different column experiments. Reactive transport modeling suggested that bacterial reduction of nitrate to nitrite, perhaps coupled with $H_2(aq)$, and abiotic $UO_2(s)$ oxidation by nitrite may occur in addition to direct enzymatic $UO_2(s)$ oxidation. A mutant strain of *T. denitrificans* that was ~50% defective in enzymatic U(IV) oxidation was used to verify a catalytic effect by bacteria. X-ray absorption spectroscopic characterization of column reaction products showed little or no evidence for a uranyl-type moiety that would indicate a surface layer of oxidized U(VI), confirming that most U was released to solution.

We previously identified two *c*-type cytochromes involved in nitrate-dependent U(IV) oxidation in *T. denitrificans* and hypothesized that *c*-type cytochromes would also catalyze Fe(II) oxidation, as they have been found to play this role in anaerobic phototrophic Fe(II)-oxidizing bacteria. Here we report on efforts to identify genes associated with nitrate-dependent Fe(II) oxidation, namely (a) whole-genome transcriptional studies [using $FeCO_3$, Fe^{2+}, and U(IV) oxides as electron donors under denitrifying conditions], (b) Fe(II) oxidation assays performed with knockout mutants targeting primarily highly expressed or upregulated *c*-type cytochromes, and (c) random transposon-mutagenesis studies with screening for Fe(II) oxidation. Assays of mutants for 26 target genes, most of which were *c*-type cytochromes, indicated that none of the mutants tested were significantly defective in nitrate-dependent Fe(II) oxidation. The non-defective mutants included the c_1-cytochrome subunit of the cytochrome bc_1 complex (complex III), which has relevance to a previously proposed role for this complex in nitrate-dependent Fe(II) oxidation and to current concepts of reverse electron transfer. A transposon mutant with a disrupted gene associated with NADH:ubiquinone oxidoreductase (complex I) was ~35% defective relative to the wild-type strain; this strain was similarly defective in nitrate reduction with thiosulfate as the electron donor. Overall, our results indicate that nitrate-dependent Fe(II) oxidation in *T. denitrificans* is not catalyzed by the same *c*-type cytochromes involved in U(IV) oxidation, nor have other *c*-type cytochromes yet been implicated in the process.

Papers and Other Products Delivered

O'Day, P. A. (2014) *Keynote:* Applications and new challenges in molecular-scale characterization of inorganic contaminants. *Goldschmidt 2014, International Geochemistry Conference*, Sacramento, CA, June 8-13.

O'Day, P. A., Chorover, J., Steefel, C., Beller, H. R., Kanematsu, M., Perdrial, N., Reinoso-Maset E., and Vasquez-Ortega A. (2014) *Invited:* Scaling of molecular processes to quantify biogeochemical reaction and transport of uranium in subsurface systems, *TES/SBR Joint Investigators Meeting*, Potomac MD, May 6-7.

Beller, H. R., Zhou, P., Legler, T. C., Chakicherla, A., Kane, S., Letain, T. E., and O'Day, P. A. (2013) Genome-enabled studies of anaerobic, nitrate-dependent Fe(II) oxidation in the chemolithoautotrophic bacterium *Thiobacillus denitrificans*, *Frontiers in Microbial Physiology and Metabolism,* 4:249. doi:10.3389/fmicb.2013.00249.

O'Day, P. A., Asta, M. P., Kanematsu, M., Steefel, C. and Beller, H. R. (2013) *Invited:* Reaction mechanisms, pathways, and transport in anaerobic abiotic and microbial U(IV)-oxide dissolution studies. *Goldschmidt 2013, International Geochemistry Conference*, Florence, Italy, August 26-30.

Asta, M. P., Kanematsu, M., Zhou, P., Beller, H. R., Traina, S., and O'Day, P. A. (2012) Mechanisms and kinetics of anaerobic abiotic and nitrate-dependent bacterial U(IV) oxidation. *Geological Society of America Annual Meeting,* November 4-7, Charlotte, NC.

Beller, H. R., Legler, T. C., Kane, S. R., O'Day, P. A., and Zhou, P. (2012) Genome-enabled studies of anaerobic, nitrate-dependent U(IV) oxidation. Oral presentation, *Goldschmidt 2012 Conference*, June 24-29, Montreal, Canada.

Zhou, P., and Beller, H. R. (2012) Investigation of genes involved in anaerobic, nitrate-dependent Fe(II) oxidation in *Thiobacillus denitrificans*. Poster presentation, *112th General Meeting of the American Society for Microbiology*, San Francisco, CA, June 2012.

O'Day, P. A., Asta, M. P., Traina, S., Beller, H. R., Zhou, P., and Steefel, C. (2012) Molecular mechanisms and kinetics of microbial anaerobic nitrate-dependent U(IV) and Fe(II) oxidation. Poster presentation, *DOE-SBR Annual Principal Investigator Meeting*, Washington DC, April 30-May 2.

Asta, M. P., O'Day, P. A., Kanematsu, M., Beller, H. R., Zhou, P., Traina, S., and Steefel, C. (2012) Kinetics of abiotic and microbial anaerobic, nitrate-dependent U(IV) oxidation. Poster presentation, *American Chemical Society Spring 2012 National Meeting,* March 25-29, San Diego, CA.

Zhou, P., and Beller, H. R. (2012) Investigation of genes involved in anaerobic, nitrate-dependent Fe(II) oxidation in *Thiobacillus denitrificans*. Poster presentation, *American Chemical Society Spring 2012 National Meeting,* March 25-29, San Diego, CA.

Zhou, P., and Beller, H. R. (2011) Different enzymes are involved in anaerobic, nitrate-dependent U(IV) and Fe(II) oxidation in *Thiobacillus denitrificans*. Poster presentation, *American Geophysical Union Fall Meeting*, San Francisco, CA, December 2011.

O'Day, P. A., Beller, H. R., Asta, M. P., Zhou, P., Traina, S., and Steefel, C. (2011) Molecular mechanisms and kinetics of microbial anaerobic nitrate-dependent U(IV) and Fe(II) oxidation. Poster presentation, *DOE-SBR Annual Principal Investigator Meeting*, Washington DC, April 26-28.