From nanowires to biofilms: an exploration of novel mechanisms of uranium transformation mediated by *Geobacter* bacteria

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Please briefly (16000 chars or less) summarize your most recent results to date:

The goal of this exploratory proposal was to elucidate the molecular mechanism(s) for uranium immobilization by *Geobacter* cells as they transitioned from planktonic cells to biofilms.

OBJECTIVE 1: Biological mechanism of U reduction in planktonic cells

The removal of U(VI) from groundwater following the in situ stimulation of metal reduction is often concomitant with substantial increases in the growth and activity of dissimilarity metal-reducing microorganisms in the family Geobacteraceae. However, despite extensive efforts to understand the mechanisms and pathways used by these bacteria to reduce U(VI), the nature of its U reductase had remained elusive for almost two decades. Furthermore, it had not even been conclusively demonstrated that *Geobacter* spp. could reductively precipitate U(VI) as U(IV) in the laboratory either. Because the energy to support the growth of *Geobacter* bacteria after in situ stimulation results from the reduction of the abundant Fe(III) oxides, a process that requires the expression of their conductive pili, we evaluated the ability of non-piliated, piliated and hyperpiliated strains of *G. sulfurreducens* to reduce U. Pili expression significantly enhanced the rates and extent of uranium immobilization and reduction per cell and prevented periplasmic mineralization. As a result, pili expression also preserved the cell's vitality and viability. Uranium preferentially precipitated along the pili and, to a lesser extent, on outer membrane redox-active foci. In contrast, the pilus-defective strains had different degrees of cell envelope mineralization, which correlated well with the outer membrane *c*-cytochrome content. X-ray absorption spectroscopy analyses demonstrated the extracellular reduction of U(VI) to mononuclear U(IV) complexed by carbon containing ligands, consistent with a biological reduction. In contrast, the U(IV) in the pilin-deficient mutant cells also required an additional phosphorous ligand, in agreement with the distinct periplasmic mineralization of uranium observed in this strain.

An insufficient knowledge of the biological mechanisms of contaminant transformation often limits the performance of *in situ* subsurface bioremediation and long-term stewardship strategies. The identification of *Geobacter*'s pili as the primary uranium reductase in these organisms provides a much-needed, fundamental mechanistic understanding of uranium reduction by *Geobacter* spp. required to design effective *in situ* bioremediation strategies. Analyses of transcript abundance for key *Geobacteraceae* genes are useful tools to predict the metabolic and physiological state of *Geobacter* bacteria during *in situ* bioremediation, yet provide no information about the biological mechanism of uranium reduction. However, similar tools could be applied to monitor the activity of conserved components of *Geobacter*'s pilus apparatus to assess the effectiveness of *in situ* bioremediation schemes. We recently published these findings in *PNAS* (1).

OBJECTIVE 2: Biological mechanism of U reduction by biofilms

At the beginning of this proposal, it was not known if biofilms were relevant in the subsurface and during *in situ* bioremediation. However, recent studies (2) at the Rifle, Colorado Integrated Field Research Challenge (IFRC) site demonstrated that field-scale addition of acetate to groundwater also stimulated the growth of *Geobacter* spp. in the sediment particles. Furthermore, their growth shifted from the groundwater to the solid phases during the field-scale acetate addition, where they outcompeted other organisms. This and the fact that the expression of *Geobacter* conductive pili, their primary U reductase, also leads to cell aggregation and biofilm formation (*3-5*), prompted us to investigate the role of biofilms in U

transformations. We began studying the kinetics of U reduction by biofilms in cell suspension assays and in reference to controls with planktonic, pili-expressing cells. While U(VI) was removed linearly during the first 12 h by the planktonic cells, the biofilms continued to remove U linearly for 24 h. Furthermore, the respiratory activities of the biofilm cells were similar in biofilms exposed to concentrations of U as high as 2.5 mM and approximately half of this activity was still detected after exposure to 5mM concentrations of U for 24h. This suggested that the biofilms were more resistant to U permeation and toxicity than planktonic cells. Scanning Electron Microscopy coupled to energy dispersive X-ray spectroscopy (SEM-EDS) revealed extensive U precipitation on the biofilm microcolonies after 24 h of exposure to 1 mM U(VI) acetate.

The U removal activities of the biofilms were similar in biofilms grown to different stages of development (24-h monolayers, 48-h microcolonies and 72-h mature biofilms), suggesting that the biofilm surface area exposed to the soluble U, rather than the thickness and structure of the biofilm, limited the removal activity. As a result, the removal activity per biomass unit was highest during the first stages of biofilm development. By contrast, XANES analyses demonstrated that the U reduction activity of the biofilms increased as the biofilms developed: from 18.5% U(IV) measured in the monolayered biofilms to 66.5 and 89% U((IV) in multilayered biofilms (microcolonies and more matured biofilms, respectively). These results demonstrate that biofilms play a role in U(VI) transformations yet their mechanism of U removal is different dependent of the biofilm stage of development: sorption by monolayered biofilms and reductive by multilayered biofilms. This mechanistic difference is consistent with our initial hypothesis that the biofilm physiology changes substantially during biofilm development.

As the development of multilayered biofilms by *G. sulfurreducens* requires the expression of its conductive pili, we studied the U removal and reduction activities of biofilms formed by a pilin-deficient mutant and its genetically complemented strains (pRG5*::pilA*). The pilin-mutant biofilms adsorbed U(VI) at rates comparable to the wild type, but could not efficiently reduce U(VI) to U(IV). However, complementation of the mutation *in trans* restored the removal and reduction activities. The results demonstrate that the, as in planktonic cells, *Geobacter* conductive pili are the primary U reductase in the biofilms. However, pili-deficient biofilms still reduce U. This could be due to the permeation of U inside the biofilm cells and its unspecific reduction by low potential electron donors of the cell envelope. Alternatively, secondary redox-active components could participate in U reduction, similarly to what we proposed for planktonic cells. We are currently analyzing EXAFS data to model the atomic coordinations of the U moiety in the wild-type and pilin-deficient mutant biofilms, which will provide insights into the mechanism of U reduction. A paper describing this work is now in preparation (*6*).

OBJECTIVE 3: Genetic analyses of biofilm formation in Geobacter sulfurreducens *and implications for U bioremediation*

We screened a library of randomly generated transposon-insertion mutants to identify biofilm-defective mutant, i.e., mutants interrupted at the monolayer stage or unable to fully develop as mature, multilayered biofilms (herein referred to as microcolonies). A paper describing this work is currently in preparation (*7*). Out of 4,000 mutants we identified 92 mutants interrupted at the monolayer stage, and 61, as microcolonies. The genes were then grouped in functional categories, as follows:

1. Electron transport: We identified mutants with a transposon insertion in genes encoding components of the pilus apparatus (*pilC* (pilus biogenesis), *pilR* (regulation of pilus synthesis and assembly), and *pilT-4* (pilus retraction)). We also identified several previously uncharacterized outer membrane *c*-cytochromes that are required for the transition from monolayer to microcolony linked to the U removal activity of *G. sulfurreducens* cells (*8*) and genes that indirectly control the expression of *c*-cytochromes. We also identified mutants

involved in hydrogenase maturation which enable the use of H_2 as an electron sink and as a mechanism of electron transfer in multilayered biofilms.

2. Cell envelope: Genes involved in the synthesis of an exopolysaccahride (EPS) matrix and of cyclopropane fatty acids (CFAs) are also required for biofilm formation and U reduction. Column studies of sediments stimulated with ethanol demonstrated increases in CFAs during the active phase of U bioremediation overlapping with the growth of *Geobacteraceae*. Thus, understanding the role of CFAs in biofilm maturation may provide biofilm-specific lipid signatures.
3. Others: We identified mutations in genes involved in DNA repair, metabolism, transport, regulation and signal transduction. Some metabolic genes show promise as biofilm-specific markers because they are not expressed in planktonic cells yet are required for biofilm development. This study helped us identify biofilm markers that can be used to predict and monitor the activity of *Geobacter* during in situ bioremediation.

REFERENCES

1. D. L. Cologgi, S. Lampa-Pastirk, A. M. Speers, S. D. Kelly, G. Reguera, Extracellular reduction of uranium via *Geobacter* conductive pili as a protective cellular mechanism. *Proc. Natl. Acad. Sci. USA* **108**, 15248 (Sep 13, 2011).
2. L. J. Kerkhof, K. H. Williams, P. E. Long, L. R. McGuinness, Phase preference by active, acetate-utilizing bacteria at the Rifle, CO Integrated Field Research Challenge Site. *Environ. Sci. Technol.*, (Jan 12, 2011).
3. G. Reguera *et al.*, Extracellular electron transfer via microbial nanowires. *Nature* **435**, 1098 (Jun 23, 2005).
4. G. Reguera *et al.*, Biofilm and nanowire production lead to increased current in microbial fuel cells. *Appl. Environ. Microbiol.* **72**, 7345 (Nov, 2006).
5. G. Reguera, R. B. Pollina, J. S. Nicoll, D. R. Lovley, Possible nonconductive role of *Geobacter sulfurreducens* pilus nanowires in biofilm formation. *J. Bacteriol.* **189**, 2125 (Mar, 2007).
6. D. L. Cologgi, A. M. Speers, B. Bullar, S. D. Kelly, G. Reguera, Immobilization and reduction of uranium during the development of *Geobacter sulfurreducens* biofilms. (in preparation) (2012).
7. D. L. Cologgi, A. Otwell, J. Rotondo, G. Reguera, Genetic analyses of biofilm formation in *Geobacter sulfurreducens*. (in preparation) (2012).
8. E. S. Shelobolina *et al.*, Importance of *c*-Type cytochromes for U(VI) reduction by *Geobacter sulfurreducens*. *BMC Microbiol.* **7**, 16 (2007).

Please briefly (7000 chars or less) describe papers and other products delivered:

- Publications:

Reguera G. (2012) *Electron transfer at the cell-uranium interface in Geobacter spp.* Biochem. Soc. Trans. 40(6), 1227-1232

Cologgi, D. L., S. Lampa-Pastirk, A. M. Speers, S. D. Kelly, **G. Reguera** (2011) Extracellular reduction of uranium via *Geobacter* conductive pili as a protective cellular mechanism. Proc. Natl. Acad. Sci. USA 108, 15248. (*Science* Editor's Choice, *Nature News*, *BBC Science News*)

Cologgi, D. L., A. M. Speers, B. Bullar, S. D. Kelly, **G. Reguera** (2013) Immobilization and reduction of uranium during the development of *Geobacter sulfurreducens* biofilms. (in preparation).

Cologgi, D. L., A. Otwell, J. Rotondo, **G. Reguera** (2013) Genetic analyses of biofilm formation in *Geobacter sulfurreducens*. (in preparation).

- Patents:

Reguera, G., D. L. Cologgi, R. M. Worden, A. Castro Forero, R. Steidl, *Methods for the reductive precipitation of soluble metals and biofilms and devices related thereto*. US and international Patent Application submitted on August 30, 2012, claiming priority from US Provisional Application No. 61/530,708, filed on September 2, 2011, and from US Patent Application Serial No. 61/558,091, filed November 10, 2011.

- Poster presentations:

Cologgi, D. L., A. M. Speers, S. Lampa-Pastirk, B. Bullard, A. Otwell, J. Rotondo, S. D. Kelly, and G. Reguera (2012) From nanowires to biofilms: an exploration of novel mechanisms of uranium transformation mediated by *Geobacter* bacteria. Department of Energy, Subsurface Biogeochemical Research (DOE-SBR) 7th annual PI meeting. Washington D.C., April 30 - May 2, 2012.

Cologgi, D. L., A. M. Speers, S. D. Kelly, G. Reguera (2011) From nanowires to biofilms: an exploration of novel mechanisms of uranium transformation mediated by *Geobacter* bacteria. Department of Energy, Subsurface Biogeochemical Research (DOE-SBR) Contractor-Grantee Workshop. Washington D.C., April 26-28, 2011.

Cologgi, D. L., A. M. Speers, B. Bullard, S. D. Kelly, G. Reguera (2010) 'From nanowires to biofilms: an exploration of novel mechanisms of uranium transformation mediated by *Geobacter* bacteria'. Department of Energy, Subsurface Biogeochemical Research (DOE-ERSP) Contractor-Grantee Workshop. Washington D.C., March 29-31, 2010.

- Invited talks:

BioCom2: Biological communication and computation workshop, Boston (Nov 8-9) (*co-organizer, conveyer and speaker*)

Department of Energy Subsurface Biogeochemical Research Annual PI's meeting, Washington DC (May 1st, 2012)

Electron transfer at the microbe-mineral interface, focused meeting of the Biochemical Society, University of East Anglia, Norwich, UK, (April 2-4, 2012)

American Chemical Society National meeting, Environmental bioinorganic Symposium, San Diego, CA, March 29th, 2012. Invited talk by G.R.

Superfund Research Program Annual Meeting (National Institutes of Environmental Health Sciences). Lexington, KY, October 24, 2011 (*conveyer and speaker*)

National Institute of Environmental Health Science, Superfund Research Program Annual Meeting, R01 grantee session, Lexington, KY (October 23rd, 2011).

Microbiology/Crop & Soil Science departments joined seminar, Michigan State University (October 6th 2011)

Science University, College of Natural Science, Michigan State University (April 2011)

National Institute of Environmental Health Science, Superfund Research Program Annual Meeting, Sustainability Research in the SRP: Progress and Benefits, session co-chair and speaker (November 12th, 2010)

College of Natural Science Board of Directors meeting, Michigan State University (November 5th 2010)

American Society for Microbiology, Michigan Branch, General meeting keynote speaker (October 9th 2010)

Congress on Industrial Biotechnology and Bioprocessing, Biological Electron Transfer and Energy Production session, Washington D.C. (June 29th 2010)

Department of Microbiology, University of Illinois (September 10th 2009)

Department of Civil Engineering, Michigan State University (November 11th 2008)
Department of Crop and Soil Sciences, Michigan State University (October 18th 2008)
Center for Nanomaterials, Michigan State University (September 18th 2008)
CMB/GEN retreat, Michigan State University (August 22th 2008)
Metabolism, Membranes, and Metalloenzymology (The 3M Interest Group), department of Biochemistry and Molecular Biology, Michigan State University (February 25th 2008)

Please provide any new notes (7000 chars or less) concerning the project:

The project ended up being more intensive than anticipated. While we originally planned to focus our work on biofilms only, it became apparent early on in the project that we first had to investigate the biological mechanism of U reduction by planktonic cells. This mechanism had remained elusive for almost two decades but was critical for our studies as a reference to understand the role of U transformations by biofilms. Because of this, we requested a no-cost extension at the end of year 2 (our final year) In the no-cost extension year, we completed all the biofilm studies that we proposed to do. Due to the limited funds, only one student was assigned to the project at any given semester. Most of the work was done by student Dena Cologgi, with student Allison Speers being partially involved in the XAS analyses and biofilm studies. The major outcomes of the work are four publications, including one in *PNAS* that received great attention, and one patent.