TOWARDS A MORE COMPLETE PICTURE: DISSIMILATORY METAL REDUCTION BY *ANAEROMYXOBACTER* SPECIES

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Manuscripts published:

1. Rademacher, L. K., C. C. Lundstrom, T. M. Johnson, R. A. Sanford, J. Zhao, and Z. Zhang. 2006. Experimentally determined Uranium isotope fractionation during reduction of hexavalent U by bacteria and zero valent iron. Environmental Science and Technology 40:6943-6948.

2. Löffler, F. E., and E. A. Edwards. 2006. Harnessing microbial activities for environmental cleanup. Curr. Opin. Biotechnol. 17:274-284.

3. Wu, Q., R. A. Sanford, and F. E. Löffler. 2006. Uranium (VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl. Environ. Microbiol. 72:3608-3614.

4. Sung, Y., K. E. Fletcher, K. M. Ritalahti, R. P. Apkarian, N. Ramos-Hernández, R. A. Sanford, N. M. Mesbah, and F. E. Löffler. 2006. *Geobacter lovleyi* Strain SZ sp. nov., a Novel Metal-Reducing and Tetrachloroethene (PCE)-Dechlorinating Bacterium. Appl. Environ. Microbiol. 72:2775-2782.

5. Löffler, F. E., R. A. Sanford, and K. M. Ritalahti. 2005. Enrichment, cultivation, and detection of reductively dechlorinating bacteria. Methods Enzymol. Vol. 397:77-111.

6. Löffler, F. E., and R. A. Sanford. 2005. Analysis of trace hydrogen metabolism. Methods Enzymol. Vol. 397:222-237.

Manuscripts accepted for publication:

7. Sanford, R. A., Q. Wu, Q., Y. Sung, S. H. Thomas, B. K. Amos, E. K. Prince, and F. E. Löffler. 2007. Hexavalent uranium supports growth of *Anaeromyxobacter dehalogenans* and *Geobacter* spp. with lower than predicted biomass yields. Environmental Microbiology. Accepted for publication.

Manuscripts submitted:

8. Amos, B. K., Y. Sung, K. E. Fletcher, T. J. Gentry, W.-M. Wu, C. S. Criddle, J. Zhou, and F. E. Löffler. 2007. Detection and quantification of *Geobacter lovleyi* strain SZ: implications for bioremediation at tetrachloroethene- (PCE-) and uranium-impacted sites. Applied and Environmental Microbiology. Submitted.

Manuscripts in preparation:

9. Thomas S. H., et al. 2007. The mosaic genome of *Anaeromyxobacter dehalogenans* strain 2CP-C suggests an aerobic common ancestor to the delta-Proteobacteria. In preparation.

10. Preston, K.E., A. Woolfitt, H. Moura, S. H. Thomas, Q.Wu, Y. Sung, J. R. Barr, and F. E. Löffler. Whole-cell MALDI-TOF MS detection and monitoring of *Anaeromyxobacter dehalogenans.* In preparation.

11. Wu, Q., S. H. Thomas, R. Wagner, G. Rodriguez, A. Massol, K. Krishnani, K. M. Ritalahti, R. A. Sanford, J. Chee-Sanford, and F. E. Löffler. 2007. Nitrous oxide respiration by facultative anaerobic myxobacteria. In preparation.

12. Wu, Q., S. H. Thomas, R. A. Sanford, K. T. Finneran, and F. E. Löffler. 2007. Phylogenetically similar *Anaeromyxobacter dehalogenans* isolates from uranium-contaminated sites exhibit distinct metal reduction capabilities. In preparation.

Poster presentations:

1. Thomas, S. H., R. D. Wagner, A. K. Arakaki, J. R. Kirby, I. Zhulin, L. Shimkets, J. Skolnick, and F. E. Löffler. 2007. Rooting the Myxococcales in the delta-Proteobacteria: genome analysis of *Anaeromyxobacter dehalogenans* strain 2CP-C. Abstract N-175. *In* Abstracts of the 107th General Meeting of the American Society for Microbiology, Toronto, Canada, May 21-25, 2007.
2. Hauer, K., M. Pavlekovic, H. Sun, S. Spring, I. Nijenhuis, H. Richnow, M. Fiandaca, B. K. Amos, K. Fletcher, K. Ritalahti, S. H. Thomas, F. Löffler, and N. Lee. 2007. Development and optimization of *in situ* detection techniques for reductively dechlorinating and metal-reducing bacteria. Abstract Q-187. *In* Abstracts of the 107th General Meeting of the American Society for Microbiology, Toronto, Canada, May 21-25, 2007.
3. Wu, Q., Thomas, S.H., R. D. Wagner, R.A. Sanford, J. Chee-Sanford, and F. E. Löffler. 2007. Nitrous oxide respiration in non-denitrifying *Anaeromyxobacter dehalogenans*. Abstract N-005. *In* Abstracts of the 107th General Meeting of the American Society for Microbiology, Toronto, Canada, May 21-25, 2007.
4. Strycharz, S. M., K. P. Nevin, T. L. Woodward, R. A. Sanford, F. E. Löffler, and D. R. Lovley. 2007. Electron Transfer to and from Potentiostat-PoisedGraphite Electrodesby Two Anaerobic, Dehalogenating Bacteria. Abstract Q-259. *In* Abstracts of the 107th General Meeting of the American Society for Microbiology, Toronto, Canada, May 21-25, 2007.
5. Thomas, S. H., Q. Wu, E. Padilla, N. M. Lee, R. A. Sanford, and F. E. Löffler. 2006. *Anaeromyxobacter dehalogenans* Strain Diversity in Uranium and Nitrate-contaminated FRC Sediment. DOE-ERSP PI Meeting, October 23-25, Oak Ridge, TN.
6. Preston, K.E., F. E. Löffler, A. Woolfitt, H. Moura, S. H. Thomas, Q.Wu, Y. Sung, J. R. Barr. 2006. Detection of substrate-specific biomarkers for metabolically versatile bacteria by whole-cell MALDI-TOF MS. American Chemical Society National Meeting, September 10-14, San Francisco, CA.
7. Chee-Sanford, J. C., L. M. Connor, R. A. Sanford, F. E. Löffler, S. H. Thomas, and G. K. Sims. 2006. Investigating anaerobic microbial processes in agricultural soils using *Anaeromyxobacter dehalogenans* as a cosmopolitan model. 11th International Symposium on Microbial Ecology - ISME-11, Vienna, Austria.
8. Thomas, S. H., M. Pavlekovic, N. Lee, M. J. Marshall, D. W. Kennedy, K. M. Ritalahti, R. A. Sanford, and F. E. Löffler. 2006. Diversification in the subsurface: Strain variation of *Anaeromyxobacter dehalogenans* in a uranium- and nitrate-contaminated subsurface environment. Abstract A87, 11th International Symposium on Microbial Ecology, ISME-11, August 20-25, Vienna, Austria.

9. Marshall, M. J., A. S. Beliaev, D. W. Kennedy, A. C. Dohnalkova, A. E. Plymale, S. H. Thomas, F. E. Löffler, R. A. Sanford, S. B. Reed, D. E. Culley, Y. Zang, D. A. Saffarini, M. F. Romine, J. M. Zacchara, and J. K. Fredrickson. Biochemical mechanisms of technetium(VII) reduction by *Shewanella oneidensis* and *Anaeromyxobacter dehalogenans*. 11th International Symposium on Microbial Ecology, ISME-11, August 20-25, Vienna, Austria.
10. Padilla, E., S. H. Thomas, Q. Wu, R. A. Sanford, and F. E. Löffler. 2006. Characterization of the microbial communities in mixed waste site sediments. Society for Advancement of Chicanos/Latino and Native Americans in Science, October 26-29, Tampa, FL.
11. Sung, Y., T. Gentry, J, Zhou, and F. E. Löffler. 2006. 16S rRNA gene-based detection of tetrachloroethene- and uranium-reducing *Geobacter lovleyi* strain SZ, abstr. Q-391. *In* Abstracts of the 105th General Meeting of the American Society for Microbiology, Orlando, FL.
12. Preston, K. E., J. R. Barr, A. Woolfitt, H. Moura, B. Amos, Y. Sung, S. Henry, F. E. Löffler. 2006. Characterization of *Anaeromyxobacter dehalogenans* and Related Metal-Reducing Bacteria by MALDI-TOF MS. DOE-ERSP PI Meeting, April 3-5, Warrenton, VA.
13. Wu, Q., S. H. Thomas, R. A. Sanford, and F. E. Löffler. 2006. Uranium (VI) reduction by *Anaeromyxobacter dehalogenans.* DOE-ERSP PI Meeting, April 3-5, Warrenton, VA.
14. Pavlekovic, M., I. Nijenhuis, F. E. Löffler, H. Richnow, N. Lee. 2006. In situ approaches for structural and functional analysis of microbial communities. AXIOM-Virtual Institute Spring School, Microbial activity in biogeochemical gradients, Leipzig, Germany (http://www.ufz.de/index.php?en=6393).
15. Preston, K. E, J. R. Barr, H. Moura, A. Woolfitt, B. Amos, Y. Sung, S. Henry, and F. E. Löffler. 2005. MALDI-TOF MS profiling of metal-reducing bacteria including *Anaeromyxobacter*, *Geobacter*, and *Desulfuromonas* species. The Joint International Symposia for Subsurface Symposia for Subsurface Microbiology and Environmental Biogeochemistry, Jackson Hole, WY.
16. Wu, Q., R. A. Sanford, F. E. Löffler. 2005. Uranium reduction by *Anaeromyxobacter* species, abstr. Q-038. *In* Abstracts of the 104th General Meeting of the American Society for Microbiology, Atlanta, GA.

Oral presentations:

Drs. Löffler and Sanford gave more than 15 invited talks at national and international meetings and presented work performed under this ERSP grant.

Background and Rational

Studies on bacterial U(VI) reduction have largely focused on the well-known metal (e.g., *Shewanella* spp. and *Geobacter* spp.) and sulfate (e.g., *Desulfovibrio* spp.) reducing bacteria. *Anaeromyxobacter dehalogenans* strain 2CP-C, and several related strains, have been isolated based on their ability to grow with 2-chlorophenol as electron acceptor (Sanford et al. 2002). Based on 16S rRNA gene sequence analysis and phenotypic characterization, the new isolates formed a novel genus, *Anaeromyxobacter*, clustered within the Myxococcales group in the delta-Proteobacteria. Hence, *Anaeromyxobacter dehalogenans* is the first myxobacterium capable of anaerobic respiration. The *Anaeromyxobacter* isolates displayed metabolic versatility and used a variety of electron donors, including acetate and hydrogen, and electron acceptors, including *ortho*-substituted halophenols, soluble and amorphous ferric iron, nitrate, nitrite, fumarate, humic substances (i.e., anthraquinone-2, 6-disulfonate or AQDS), or oxygen to support growth. Remarkable was the rapid reduction of soluble and insoluble forms of ferric iron to ferrous iron by all *Anaeromyxobacter* isolates (He et al. 2003) suggesting that these organisms are good metal reducers.

A 16S rRNA gene-based community analysis of Fe(III)-reducing enrichment cultures obtained from uranium-contaminated sediment collected at the Integrated Field-Scale Subsurface Research Challenge (IFC) site (i.e., the FRC site) near Oak Ridge, TN, suggested the predominance of organisms related to the genus *Anaeromyxobacter* (Petrie et al. 2003). Biostimulation push-pull tests at the Oak Ridge IFC site yielded additional evidence of metal-reducing microorganisms in acidic subsurface sediments associated with both the *Anaeromyxobacter* and *Geobacter* genera (North et al. 2004). In column studies performed with Oak Ridge IFC site materials, *Anaeromyxobacter* spp. and *Geobacter* spp. were the predominant microbes in the iron-reducing zones (Lloyd 2007). These findings suggested that *Anaeromyxobacter* spp. may contribute to reductive dechlorination, reduction of oxidized nitrogen species and U(VI) immobilization at the Oak Ridge IFC site.

Objectives

The overarching goal of this 3-year project was to explore hexavalent uranium, U(VI), reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C, and to demonstrate that *Anaeromyxobacter* spp. contribute to U(VI) reduction and immobilization at the Oak Ridge IFC site. To accomplish these goals, the following objectives were addressed:

(1) Explore U(VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C and characterize *Anaeromyxobacter* physiology as it relates to U(VI) metabolism.

(2) Measure growth yields of *Anaeromyxobacter dehalogenans* strain 2CP-C with U(VI) as electron acceptor and perform a comparative analysis with other metal reducers.

(3) Obtain *Anaeromyxobacter* isolates from the Oak Ridge IFC site.

(4) Design 16S rRNA gene-targeted PCR primers to specifically detect and quantify *Anaeromyxobacter* spp. in pure cultures, consortia, and environmental samples.

(5) Assess *Anaeromyxobacter* spp. distribution and abundance at the Oak Ridge IFC site.

(6) Explore the contributions of *Geobacter lovleyi* strain SZ-type organisms to contaminant detoxification at the Oak Ridge IFC site.

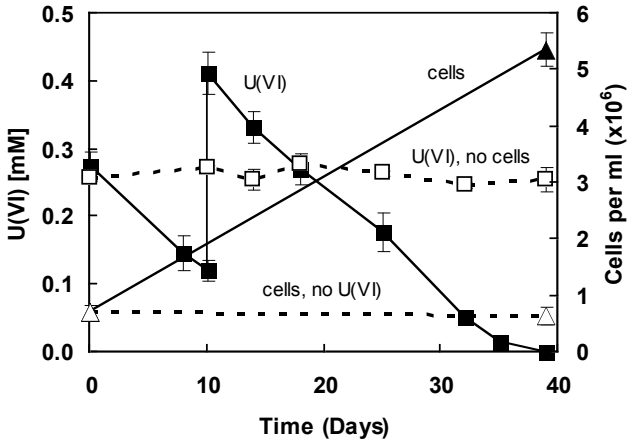
Summary of Results

Objective 1. Explore U(VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C and characterize *Anaeromyxobacter* physiology as it relates to U(VI) metabolism

Cell suspensions of fumarate-grown *Anaeromyxobacter dehalogenans* strain 2CP-C cells readily reduced U(VI) to U(IV). In these cultures, U(VI) was reduced by 90% within 8 hours of incubation. Suspensions of washed, fumarate-grown 2CP-C cells amended with acetate and hydrogen reduced U(VI) following a lag time of <3 days, while loss of soluble U(VI) in the killed controls was negligible.

Subsequent experiments explored the ability of strain 2CP-C to grow at the expense of U(VI) reduction. Monitoring the increase in 16S rRNA gene copy numbers using quantitative real-time PCR (qPCR) in cultures provided with U(VI) as an electron acceptor demonstrated growth (Fig. 1).

Figure. 1. U(VI) reduction coupled to growth in cultures of *A. dehalogenans* strain 2CP-C amended with H_2 as electron donor. The closed squares indicate the U(VI) concentration in growing cultures, and the open squares represent U(VI) concentrations in cell-free controls. The triangles indicate the cell numbers in culture vessels amended with U(VI) (closed symbols) and cultures lacking U(VI) (open symbols). At day 10, the U(VI)-reducing cultures received an additional 0.29 mM of uranyl carbonate. The results are the averages of triplicate cultures with error bars indicating the standard deviation.



Growth with U(VI) required the presence of hydrogen as electron donor and no U(VI) reduction occurred in cultures amended with acetate as electron donor (Fig. 2).

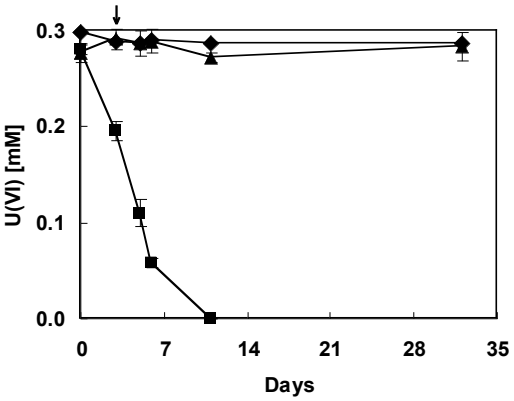


Figure 2. U(VI) reduction in cultures amended with acetate, nitrate, and uranyl carbonate ± hydrogen. ■, + hydrogen; ▲, no hydrogen; and ◆, no cells. The culture vessels were inoculated with acetate/nitrate-grown 2CP-C cells. Nitrate reduction in live cultures was complete after 3 days as indicated by the arrow. The data points represent the averages of replicate cultures, with error bars showing the standard deviation.

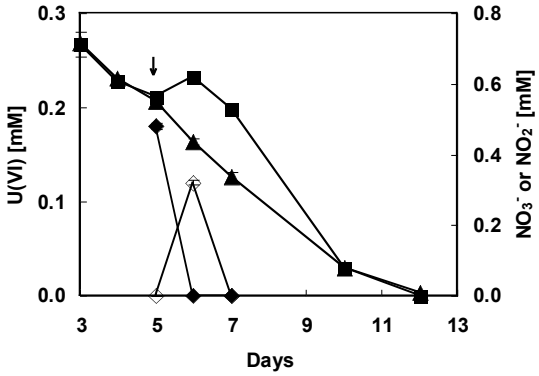
This was a surprising finding because *A. dehalogenans* spp. use acetate and hydrogen as electron donors for the oxidation of other growth-supporting electron donors. The reasons why strain 2CP-C cannot couple acetate oxidation to U(VI) reduction under growth conditions are unclear but could be related to energetic reasons. H_2 oxidation yields 13 kJ per mole of electrons more free energy ($\Delta G°'$) than acetate oxidation. Even under environmentally relevant conditions (e.g., in saturated subsurface environments), H_2 oxidation yields about 7 kJ per mole of electrons more free energy ($\Delta G'$) than acetate oxidation. Since the free energy associated with U(VI) reduction may be quite minimal depending on the pathway, this extra energy from H_2 could be sufficient to allow the reaction to proceed, while with acetate the available free energy is insufficient to drive the reaction in strain 2CP-C. Members of the extensively studied *Geobacter* group do use acetate as an electron donor for U(VI) reduction, indicating that this process is feasible and is not constrained entirely by energetics (Lloyd et al. 2002).

Many uranium-impacted sites contain co-contaminants such as nitrate and chloroorganic compounds, or contain other electron acceptors that may interfere with U(VI) reduction. Hence, the effects of other oxidants on U(VI) reduction were explored in cultures of *Anaeromyxobacter dehalogenans* strain 2CP-C.

Nitrate. *Anaeromyxobacter* spp. grow with nitrate as electron acceptor. Nitrate is reduced via nitrite to stoichiometric amounts of ammonium in a process known as dissimilatory nitrate reduction to ammonium (DNRA). Hence, the effects of nitrate on U(VI) reduction were explored in more detail. Freshly inoculated (2%, vol/vol) strain 2CP-C cultures completely reduced 0.5 mM nitrate within 3 days of incubation with acetate provided as electron donor. U(VI) reduction occurred 1 day after uranyl carbonate and hydrogen were added to the acetate/nitrate-grown cultures (Fig. 3). The addition of nitrate caused an apparent increase in soluble U(VI) concentration (Fig. 3). The increase in U(VI) concentration coincided with the intermediate formation of nitrite suggesting that nitrate-reducing conditions are

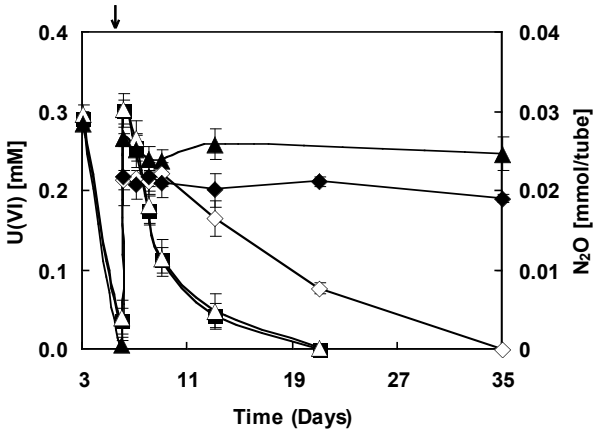
associated with U(IV) oxidation. Following complete consumption of nitrate and nitrite, U(VI) reduction resumed (Fig. 3).

Figure 3. Effect of nitrate on U(VI) reduction. ■, U(VI) + nitrate; ▲, U(VI), no nitrate, ◆, nitrate; and ◇, nitrite. Strain 2CP-C was grown with acetate/nitrate and complete nitrate reduction had occurred by day 3 when the cultures were amended with uranyl carbonate and hydrogen. The arrow indicates the addition of 0.5 mM nitrate following the onset of U(VI) reduction. Results are the averages of replicate cultures, with error bars showing the standard deviation.



N$_2$O. The genome analysis of strain 2CP-C revealed the presence of a complete *nosZ* gene cluster (Thomas et al. 2007). This was unexpected because bacteria with the DNRA pathway, cannot reduce nitrite to NO, and hence, are not dentrifiers. Interestingly, strain 2CP-C grew with N_2O as electron acceptor producing dinitrogen gas indicating that *nosZ* is fully functional (Wu et al. 2007). N_2O is a strong oxidant and is formed in the denitrification process. Since nitrate is a co-contaminant at the Oak Ridge IFC and denitrification will likely occur at the site, we explored the effects of N_2O on U(VI) reduction in cultures of strain 2CP-C. Acetate/N_2O-grown cultures that had completely reduced 1.4 mM N_2O (3 days following inoculation) were amended with 0.3 mM uranyl carbonate. Within 2 days, U(VI) was reduced and triplicate cultures received an additional feeding of uranyl carbonate. Another set of triplicate cultures received uranyl carbonate and 1.4 mM N_2O (nominal concentration) following the onset of U(VI) reduction. Figure 4 shows that U(VI) reduction occurred at similar rates in the absence and presence of N_2O suggesting that N_2O has no effect on U(VI) reduction in cultures of strain 2CP-C.

Figure 4. Effect of N_2O on U(VI) reduction. ■, U(VI) no N_2O; △, U(VI) + N_2O; ▲, U(VI), killed cells; ◇, N_2O; and ◆, N_2O, killed cells. Strain 2CP-C was grown with acetate/N_2O. N_2O reduction was complete at day 3, when uranyl carbonate and hydrogen were added. The arrow indicates when the cultures were respiked with uranyl carbonate. N_2O was added after the onset of U(VI) reduction. Results are the averages of triplicate cultures, with error bars showing the standard deviation.

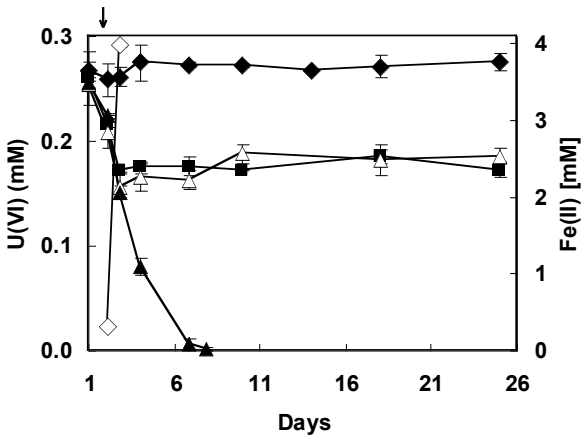


We also tested the abiotic reoxidation of U(IV) by N_2O. Negligible amounts of UVI) were formed in serum bottles containing reduced U(IV) and amended with N_2O after 15 hours of incubation. In contrast, 65% of the reduced U(IV) were reoxidized to V(VI) over the same time period when oxygen (i.e., air) was added to the vessels.

Ferric iron. The effect of ferric citrate was explored in acetate/fumarate-grown cultures that had consumed all fumarate (1 day following inoculation). In the presence of hydrogen, U(VI) was readily reduced without apparent lag phase but the addition of ferric citrate caused the immediate cessation of U(VI) reduction (Fig. 5). In contrast, complete Fe(III)-to-Fe(II) reduction occurred within 1 day following the addition of ferric citrate suggesting that the cells were metabolically active. In a parallel experiment, sodium citrate, instead of ferric citrate, was added following the onset of U(VI) reduction. The addition of citrate alone was sufficient to prevent U(VI) reduction (Fig. 5), suggesting that ferric iron was not the cause for the observed inhibition.

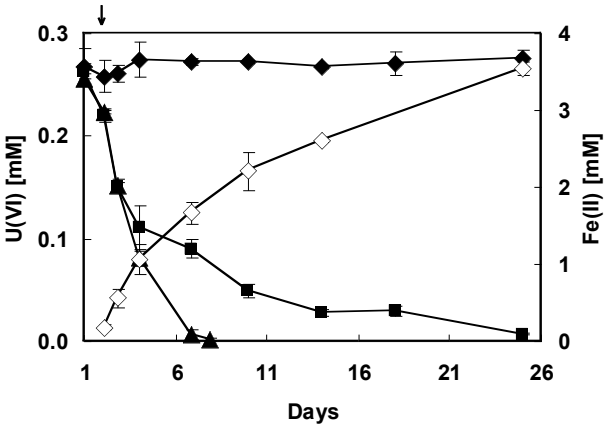
Figure 5. Effects of ferric citrate and citrate on U(VI) reduction. ■, U(VI) + ferric citrate; ▲, U(VI), no additions; △, U(VI) + citrate; ◆, U(VI) + killed inoculum; and ◇, Fe(II).

Strain 2CP-C cultures were grown with acetate/fumarate. Fumarate was consumed after 1 day, and then the cultures were amended with uranyl carbonate and hydrogen. The arrow indicates the addition of ferric citrate or sodium citrate to duplicate sets of cultures following the onset of U(VI) reduction. The data shown represent averaged values of replicate cultures, with error bars showing the standard deviation.



To further explore the effects of ferric iron on U(VI) reduction, hydrous ferric oxide (HFO) was added to U(VI)-reducing cultures. The addition of Fe(III) oxide slowed down the rate of U(VI) reduction relative to control cultures not receiving HFO; however, U(VI) reduction proceeded to completion in the presence of HFO (Fig. 6).

Figure 6. Effect of amorphous Fe(III) oxide on U(VI) reduction. ■, U(VI) + amorphous Fe(III) oxide; ▲, U(VI), no amorphous Fe(III) oxide; ◆, U(VI), killed cells; and ◇, Fe(II). Acetate/fumarate-grown strain 2CP-C cultures consumed fumarate within 1 day, at what time the cultures were amended with uranyl carbonate and hydrogen. The arrow indicates the addition of amorphous Fe(III) oxide. The data points represent the averages generated with replicate cultures, with error bars showing the standard deviation.



Concomitant with U(VI) reduction, Fe(II) was formed suggesting that both, ferric iron and U(VI), were reduced simultaneously. The addition of amorphous iron increased the time required to completely reduce the initial amount of U(VI) about 3-fold compared to cultures not receiving amorphous ferric iron (Fig. 6). To verify that the loss of U(VI) was due to microbial reduction, U(VI) sorption to Fe(III) oxide under the culture conditions applied was evaluated. Only a small amount (<2%) of the total U(VI) sorbed to 5 mM (nominal concentration) amorphous Fe(III) oxide following a 10-day incubation period suggesting that the loss of U(VI) in the live cultures was due to reduction by strain 2CP-C rather than physical (abiotic) sorption processes. The medium pH remained at 7.1 over the 10-day incubation period suggesting that no pH-induced changes in the surface characteristics of the Fe(III) oxide occurred.

2-Chlorophenol (2-CP). The effects of 2-CP on U(VI) reduction were initially explored in acetate/nitrate-grown cultures that had consumed all the provided nitrate. U(VI) was reduced at similar rates in the 2-CP-amended cultures and the controls lacking 2-CP, suggesting that 2-CP did not influence U(VI) reduction. 2-CP was dechlorinated to phenol following a 5-day lag phase in the cultures pregrown with nitrate. A second, independent experiment with cultures that had consumed 0.2 mM 2-CP confirmed that 2-CP and U(VI) were reduced concomitantly, and the presence of 2-CP had no effect on U(VI) reduction rates. In the 2-CP-pregrown cultures, phenol was produced without a lag time following the addition of 2-CP. Similarly, U(VI) had no effect on 2-CP dechlorination to phenol, and both, 2-CP and U(VI) were reduced simultaneously.

Fumarate did not inhibit U(VI) reduction, and both fumarate and U(VI) reduction occurred concomitantly.

In the experiments described above with live, hydrogen-amended cultures, U(VI) was reduced without an apparent lag phase following its addition to nitrate-, ferric iron-, 2-CP-, or fumarate-grown cells. These observations suggest that anaerobically grown cells of strain 2CP-C possess a constitutive pathway (or pathways) for U(VI) reduction.

A. dehalogenans strain 2CP-C grew at the expense of U(VI) reduction. This is a relevant observation because growth-linked, metabolic processes are easier to control and manage in the field, and hence, are more desirable than co-metabolic processes where the organisms gain no benefit (i.e., do not gain maintenance energy or sustain growth) from contaminant transformation (Löffler and Edwards 2006).

Objective 2. Measure growth yields of *Anaeromyxobacter dehalogenans* strain 2CP-C with U(VI) as electron acceptor and perform a comparative analysis with other metal reducers

For monitoring growth, cell numbers were estimated from rRNA gene copy numbers determined with qPCR. Previous work has demonstrated that qPCR is an accurate and reproducible tool with a large dynamic range to determine *A. dehalogenans* 16S rRNA gene copy numbers (Ritalahti et al., 2006). To verify the accuracy of the qPCR approach, microscopic cell counts were compared with qPCR cell number estimates. Figure 7 shows that direct microscopic cell counts matched the qPCR data closely (i.e., $8.03 \times 10^7 \pm 2.28 \times 10^7$ and $8.35 \times 10^7 \pm 6.06 \times 10^6$ cells per ml, respectively) in early stationary phase cultures. The non-parametric (Mann Whitney) test (95% confidence interval, $p>0.05$) indicated that acridine orange direct counts (AODC) and qPCR estimates were not statistically different during early stationary phase. These results indicate that the qPCR approach accurately determined bacterial cell numbers and provided information for growth yield calculations.

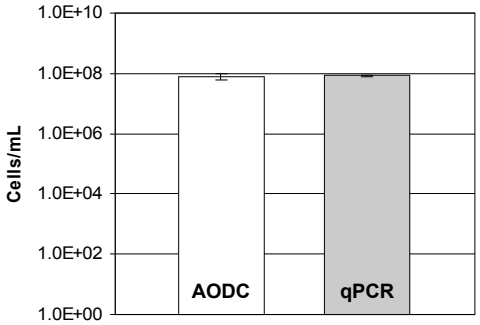


Figure 7. Comparison of microscopic counts (white bar) and qPCR (grey bar) for estimating *A. dehalogenans* cell numbers. The data shown are the averages of six qPCR replicates and 11 acridine orange direct counts (AODC) replicates (two dilutions of each) with error bars indicating the standard deviations.

Anaeromyxobacter dehalogenans strain 2CP-C reduced 0.57 ± 0.02 mM U(VI) when fed acetate and H_2, and the cell numbers increased from $0.70 \pm 0.14 \times 10^6$ (cells introduced with the inoculum) to $5.36 \pm 0.30 \times 10^6$ per ml of culture fluid (Fig. 2). Growth depended on the presence of U(VI) and no cell increase occurred in cultures that did not receive U(VI). U(VI) reduction required live cells, and no

U(VI) reduction occurred in control cultures without cells (Fig. 2). Growth yields of 7.7 ± 0.95, 8.2 ± 0.032, and 8.6 ± 0.081 x 10^6 cells produced per μmol of U(VI) reduced were determined in triplicate cultures in three independent experiments (Table 1). To evaluate the growth efficiency with uranium, the cell yields of *Anaeromyxobacter dehalogenans* strain 2CP-C obtained with different electron acceptors were compared. Fumarate-grown cells provided a reference standard to correlate cell yields determined by dry weight measurements with cell yields determined by qPCR (Table 1). On average, 0.155 ± 0.02 g of cell biomass was produced per mole of electrons transferred to U(VI). This growth yield is at least one order of magnitude lower than those measured for fumarate, ferric citrate, 2-chlorophenol, or nitrate (Table 1). In addition to the lower biomass yields, *Anaeromyxobacter dehalogenans* strain 2CP-C grew considerably slower with U(VI) as electron acceptor compared to cultures amended with fumarate, nitrate, or 2-CP as growth-supporting electron acceptors.

Table 1. Comparison of cell yields of *Anaeromyxobacter dehalogenans* strain 2CP-C grown on different electron acceptors. Cell numbers were determined by analysis of 16S rRNA gene copy numbers using qPCR. The data represent the mean and standard deviation of triplicate samples.

Electron acceptor	Electron acceptor reduced		Growth yield				Cell yield [a] (dry weight)
	(μmole/ml)	(μmole e^- /ml)	Cells/ml (x 10^7)	Cells/μmole U(VI) reduced (x 10^6)	Cells/μmole e^- transferred to EA (x 10^7)	Dry weight g/ml (x 10^{-5})	g/mole e^-
Fumarate	5.29 ± 0.13	10.58 ± 0.27	158 ± 10.9	ND	14.9 ± 1.2	6 ± 0.0	5.67 [c]
Ferric iron [b]	10.04 ± 0.21	10.04 ± 0.21	62.8 ± 1.45	ND	6.26 ± 0.09	ND	2.38 [c]
2-CP	0.93 ± 0.03	1.86 ± 0.06	9.56 ± 0.2	ND	5.16 ± 0.21	ND	1.96 [c]
Nitrate	1.60 ± 0.07	12.8 ± 0.56	222 ± 12	ND	17.3 ± 0.17	ND	6.65 [c]
U(VI) (A) [d]	0.25 ± 0.01	0.50 ± 0.02	0.192 ± 0.034	7.7± 0.94	0.383 ± 0.047	ND	0.146 [c]
U(VI) (B) [d]	0.57 ± 0.02	1.13 ± 0.04	0.467 ± 0.027	8.2± 0.32	0.41 ± 0.016	ND	0.156 [c]
U(VI) (C) [d]	0.24 ± 0.02	0.48 ± 0.03	0.211 ± 0.032	8.6± 0.82	0.43 ± 0.041	ND	0.164 [c]

ND, not determined

[a] Cell yield is reported per mole of electrons transferred to the electron acceptor based on fumarate reduction to succinate, Fe (III) reduction to Fe (II), 2-CP reduction to phenol, nitrate reduction to ammonium, and U(VI) reduction to U(IV).

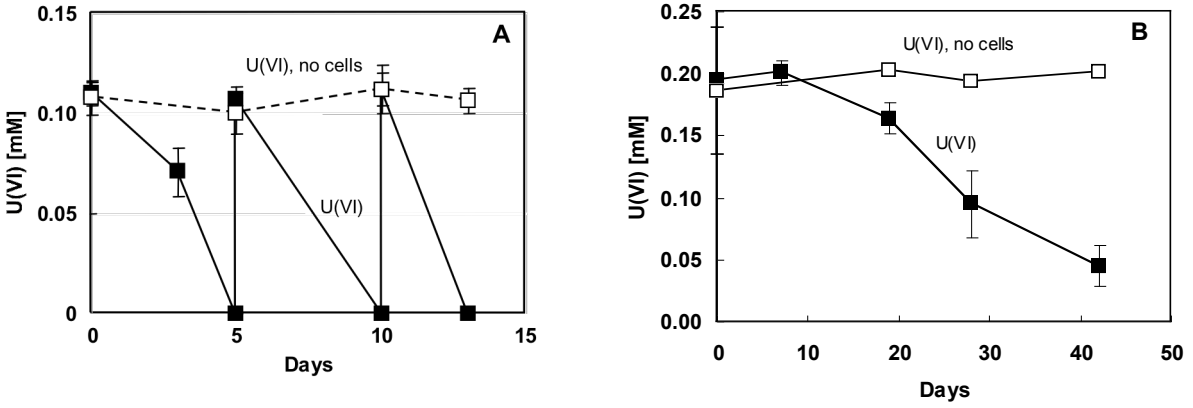
[b] Soluble ferric iron was provided as ferric citrate. No growth occurred in medium that received citrate alone (data not shown).

[c] Cell yield estimates are calculated by multiplying the number of cells per μmole of electrons transferred to the electron acceptor (column 5) times the dry weight of a single cell (3.80 ± 0.27 x 10^{-14} g/cell) estimated for fumarate-grown cells.

To compare the growth yields of *A. dehalogenans* strain 2CP-C with other members of the delta-proteobacteria, U(VI) reduction and the cell increase linked to U(VI) reduction were monitored in

cultures of two *Geobacter* species. Freshly inoculated cultures of *Geobacter lovleyi* strain SZ completely reduced 100 µM U(VI) with H_2 as an electron donor and 5 mM lactate as carbon source in 5 days. Subsequent feedings of 100 µM U(VI) were reduced in less than 5 days (Fig. 8A). U(VI) reduction in *Geobacter lovleyi* cultures was also supported by acetate as the sole source of reducing equivalents; however, U(VI) reduction occurred at lower rates compared to the H_2-amended cultures (not shown). Figure 8B shows that *G. sulfurreducens* reduced U(VI) with acetate as electron donor, although the rates of reduction were slower than those observed in *G. lovleyi* cultures amended with hydrogen. No U(VI) reduction occurred in abiotic control cultures over the respective incubation periods.

Figure 8. U(VI) reduction by *G. lovleyi* with H_2 as electron donor (A), and *G. sulfurreducens* with acetate as electron donor (B). Closed symbols indicate the U(VI) concentration in growing cultures, and the open symbols represent U(VI) concentrations in cell-free controls. The results are the averages of triplicate cultures with error bars indicating the standard deviation.



qPCR analysis demonstrated that both *Geobacter* species grew with U(VI) as electron acceptor. In cultures of *G. lovleyi*, the cell number increased from 1.53 ± 0.03 x 10^6 per ml (cells introduced with the inoculum) to 6.0 ± 0.14 x 10^6 per ml following the consumption of 300 µM U(VI). Similarly, in cultures of *G. sulfurreducens*, the cell number increased from 1.32 ± 0.27 x 10^5 per ml to 2.5 ± 1.04 x 10^6 per ml following the consumption of 150 µM U(VI). Hence, 1.49 ± 0.06 x 10^7 *Geobacter lovleyi* cells and 1.58 ± 0.71 x 10^7 *Geobacter sulfurreducens* cells were produced per µmole of U(VI) reduced. In control cultures without U(VI), the cell numbers did not increase over the incubation periods, and the *G. lovleyi* cultures contained 1.49 ± 0.41 x 10^6 cells per ml and of the *G. sulfurreducens* cultures contained 0.56 ± 0.23 x 10^5 cells per ml at the end of the incubation. The cell yields for the three organisms (i.e., *A. dehalogenans*, *Geobacter lovleyi*, and *Geobacter sulfurreducens*) were within a factor of two, indicating similar amounts of energy were gained from U(VI) reduction.

The comparison of *Anaeromyxobacter* cell yields with different electron acceptors showed that the

efficiency of growth coupled to U(VI) reduction is significantly lower compared to other electron acceptors (Table 1). When expressed as grams of biomass produced per mole of electrons consumed in electron acceptor reduction, the yield with U(VI) is at least ten times lower than those obtained with fumarate-, ferric citrate-, 2-CP-, and nitrate-grown cultures. This low yield cannot be explained by the free energy available from U(VI) reduction to U(IV). An analysis of the free energy (ΔG') under the experimental conditions applied indicates that this reduction reaction is energetically equal to fumarate reduction, and even nitrate reduction to ammonium does not yield significantly more energy on a per electron basis (Table 2).

Table 2. Free energy changes associated with different possible reactions involving U(VI) and other electron acceptors at different pH values. Shaded values indicate energetically unfavorable reactions.

Reaction	ΔG°' (kJ/mole e^-)	ΔG (kJ/mole e^-) [a]		
		pH 6	pH 7	pH 8
U(VI) → U(IV)				
$0.5\ UO_2(CO_3)_2^{-2} + 0.5\ H_2 = 0.5\ UO_2 + HCO_3^-$	-51.2	-48.3	-48.3	-48.4
$0.5\ UO_2(CO_3)_3^{-4} + 0.5\ H^+ + 0.5\ H_2 = 0.5\ UO_2 + 1.5\ HCO_3^-$	-45.8	-51.5	-48.6	-45.9
$0.5\ UO_2(CO_3)_2^{-2} + 0.125\ CH_3COO^- + 0.5\ H_2O = 0.5\ UO_2 + 0.125\ H^+ + 1.25\ HCO_3^-$	-39.55	-40.3	-41	-41.7
$0.5\ UO_2(CO_3)_3^{-4} + 1.375\ H^+ + 0.125\ CH_3COO^- + 0.5\ H_2O = 0.5\ UO_2 + 1.75\ HCO_3^-$	-34.15	-43.5	-41.3	-39.2
U(VI) → U(V)				
$UO_2(CO_3)_3^{-4} + 2\ H^+ + 0.5\ H_2 = UO_2^+ + 3HCO_3^-$	19.15	-26.5	-15.1	-3.8
$UO_2(CO_3)_2^{-2} + H^+ + 0.5\ H_2 = UO_2^+ + 2HCO_3^-$	8.35	-20.2	-14.5	-8.9
$UO_2(CO_3)_3^{-4} + 1.875\ H^+ + 0.125\ CH_3COO^- + 0.5\ H_2O = UO_2^+ + 3.25\ HCO_3^-$	30.8	-18.5	-7.8	2.9
$UO_2(CO_3)_2^{-2} + 0.875\ H^+ + 0.125\ CH_3COO^- + 0.5\ H_2O = UO_2^+ + 2.25\ HCO_3^-$	20	-12.2	-7.2	-2.2
$UO_2(CO_3)_3^{-4} + 0.5\ H_2 = UO_2(CO_3)_3^{-5} + H^+$	36.55	42.3	36.6	30.8
$UO_2(CO_3)_2^{-2} + HCO_3^- + 0.5\ H_2 = UO_2(CO_3)_3^{-5} + 2\ H^+$	25.75	48.6	37.2	25.7
Dismutation of U(V) → U(IV) and U(VI)				
$2UO_2^+ + 3HCO_3^- = UO_2 + UO_2(CO_3)_3^{-4} + 3\ H^+$	-129.9	-67.1	-84.2	-101.3
$2UO_2(CO_3)_3^{-5} + 3\ H^+ = UO_2 + UO_2(CO_3)_3^{-4} + 3HCO_3^-$	-127.6	-204.7	-187.6	-170.5
Other electron accepting processes				
$0.5\ Fe_2O_{3\ (HFO)} + 2\ H^+ + 0.5\ H_2 = Fe^{+2} + 1.5\ H_2O$	-38.79	-73	-61.6	-50.2
α-$FeO(OH)_{goethite} + 2\ H^+ + 0.5\ H_2 = Fe^{+2} + 2H_2O$	17.05	-17.2	-5.8	5.6
$0.125\ SO_4^{-2} + 0.125\ H^+ + 0.5\ H_2 = 0.125\ HS^- + 0.5\ H_2O$	-24.01	-14.7	-14.0	-13.3
0.5 Fumarate + 0.5 H_2 = 0.5 Succinate	-43.0	-51.8	-51.8	-51.8
$0.125\ NO_3^- + 0.5\ H_2 + 2\ H^+ = 0.125\ NH_4^+ + 0.375\ H_2O$	-75.0	-67.8	-69.3	-70.7

[a] Free energy changes at 25°C were calculated as described (Brooks et al. 2003, Fredrickson et al. 2003, Guillaumount et al. 2003, Roden 2003; Thauer et al. 1977) and based on the following concentrations: U(VI) = 1 mM, U(V) = 0.1 mM, HCO_3^- = 10 mM, Acetate = 10 mM, H_2 = 10,000 ppmv, Fe(II) = 0.01 mM, fumarate = 10 mM, succinate = 0.1 mM, nitrate = 1 mM, ammonium = 1 mM, sulfate = 1mM, hydrogen sulfide = 0.1 mM.

A reason for the low energy efficiency may be the toxicity of the UO_2 mineral as it accumulates in the periplasmic space (Liu et al. 2002b, Liu et al. 2002a, Lloyd, 2003) or on the outside of the cell (Lovley

and Phillips 1992, Liu et al. 2002b, Liu et al. 2002a, Marshall et al. 2006). The biochemistry of U(VI) reduction and the mechanism(s) of UO_2 inhibition are poorly understood, but it is reasonable to conceptualize an energy cost associated with UO_2 detoxification to merely permit the continuation of cell function. Extensive formation of an extracellular polymeric substance (EPS) associated with the UO_2 particles was observed in U(VI)-reducing *Shewanella* cultures (Marshall et al. 2006), suggesting that U(VI) reduction comes at an extra cost. *Anaeromyxobacter* spp. and *Geobacter* spp. may also produce EPS during growth with U(VI), consuming energy otherwise available for growth, which may explain the lower-than-expected growth yields. Alternatively, the microorganisms may not gain energy from a two electron transfer from U(VI) to U(IV) but only from a less-energy yielding reaction involving a single electron reduction of U(VI) to U(V) (Table 2). In a recent study with *G. sulfurreducens*, evidence of a pentavalent intermediate during U(VI) reduction to UO_2 was obtained using X-ray absorption fine structure (EXAFS) spectroscopy (Renshaw et al. 2005). This single electron reduction would be analogous to the reduction of ferric iron to ferrous iron and yields about one third of the free energy associated with a two electron transfer (i.e., U(VI) to U(IV)) (Table 2). U(V) is unstable and dismutates readily to U(VI) and U(IV) (Renshaw et al., 2005). This dismutation reaction releases a considerable amount of free energy, but it is unlikely that bacteria are capable of capturing energy from this spontaneous process for cellular metabolism. Thus, the free energy available to the bacterial cell from U(VI) to U(IV) reduction at pH 7 (assuming $UO_2(CO_3)_2^{-2}$ as the major solution species at pH 7) would only be -14.5 kJ per mol of electrons transferred with H_2 as the electron donor (Table 2). Such a pathway could account for the low cell yield observed with *A. dehalogenans*, *G. lovleyi*, and *G. sulfurreducens* under U(VI)-reducing conditions. Since the growth yields with different electron acceptors are directly proportional to the free energy released in the redox reactions, the cell yields also increase as the TEAPs become more energetically favorable (Rittman and McCarty 2001). The free energy associated with U(VI) to U(V) reduction is comparable to that available to sulfate reducers ($\Delta G^{\circ\prime}$ = -24 kJ/mole of electrons) when growing with H_2 as the electron donor (Table 2) (Thauer et al. 1977). Sulfate reduction yields considerably less biomass than nitrate, fumarate, or Fe(III) reduction. For example, *Desulfotomaculum acetoxidans* has an average cell yield of 0.82 g cells per mole of electrons accepted when growing with acetate and sulfate (Renshaw et al. 2005). This organism has only a slightly higher cell yield with sulfate than the U(VI) reducers measured here, and the energy available from sulfate reduction is similar to that obtained from U(VI) reduction to U(V). Thus, it is possible that energy availability affects the cell yield associated with metabolic U(VI) reduction, and a single electron reduction to U(V) could explain the lower than expected biomass yields. It is, of course, also sensible that a combination of both the cost of EPS formation and the low energy capture from a single electron transfer accounts for the low cell yields with U(VI) as electron acceptor.

Objective 3. Obtain *Anaeromyxobacter* isolates from the Oak Ridge IFC site.

Several research groups characterizing microbial communities native to Oak Ridge IFC site soil reported 16S rRNA gene sequences most closely related to *Anaeromyxobacter* spp. (Petrie et al. 2003, North et al. 2004, Lloyd 2007, M. Fields et al. unpubl. results, F. Löffler et al. unpubl. results). Oak Ridge IFC site soil samples from Area 1 (FB089-01-21and FW034) and Area 3 (FWB113-08-01, near FW029) were kindly provided by D. Watson. Microcosms were established in 60-ml vials inside an anaerobic chamber containing N_2/H_2 (97/3, vol/vol) as described (Löffler et al. 1996, 1997a,b). The soil samples were homogenized in a sterile 500 ml plastic container. Each sterile 60-ml serum bottle received approximately 3 grams of soil and basal salts medium containing 20 mM MES [2-(N-morpholino)ethanesulfonic acid and adjusted to pH 5, 5.5, and 6 with with 6M HCl or 5M NaOH. Microcosms established at neutral pH contained 30 mM bicarbonate buffer adjusted to pH 7 with CO_2. All microcosms were amended with 5 mM acetate or 0.1 mM acetate and H_2 (2 ml) as electron donors and had a total volume of 20 or 30 ml. The microcosms were amended with ferric citrate (10 mM), HFO (10 mM, nominal concentration), sodium nitrate (1 mM), N_2O (2 ml or 82 μmol), or 2-chlorophenol (0.1 mM) as electron acceptors. N_2O microcosms were flushed with a sterile stream of helium gas for 2 minutes prior to the addition of N_2O gas. The serum bottles with 2-CP were closed with Teflon-lined butyl rubber stoppers; all other containers were closed with black butyl rubber stoppers and then sealed with aluminum crimps. Duplicate microcosms were established for each treatment and incubated at 35°C. Additional microcosms established with soil from Area 1 (FWB 030, FWB 302, FB 061) and amended with HFO were provided by Dr. J. Kostka's laboratory, Florida State University.

Active microcosms showing electron acceptor consumption relative to killed control microcosms were transferred (2%, vol/vol) three times to the same media. After three transfers, active enrichment cultures were transferred to media amended with acetate as electron donor and 10 mM ferric citrate or 10 mM fumarate as electron acceptor. Enrichment cultures reducing both ferric citrate and fumarate were selected for isolation through repeated dilution-to-extinction series (with 10^{-1} to 10^{-10} dilution series) in liquid medium. Parallel dilution series were established in 20-ml vials using the same media (10 ml) amended with 5 mM acetate, 5 mM fumarate and 0.6% (w/w) low-melting agarose as described (Löffler, Sanford et al. 2005). Isolated colonies, which formed after about 2 weeks of incubation, were picked, transferred to 2 ml of fresh medium, and new dilution-to extinction series were established. This process was repeated three times to verify culture purity. Each culture was also transferred to medium amended with 0.1 mM 2-CP and 5 mM acetate to verify dechlorination activity expected of *Anaeromyxobacter* spp.

The enrichment procedure yielded one isolate from sediment of FRC FW034 (Area 1) and 10 isolates from Area 1 soil materials. All 11 isolates share greater that 99% 16S rRNA gene sequence similarity with each other and were most closely related to *Anaeromyxobacter dehalogenans* (Fig. 9). Based on 16S

rRNA gene sequence differences in signature regions, the new Oak Ridge IFC site isolates could be divided into three groups (highlighted in grey in Fig. 9). Another *Anaermyxobacter* isolate (strain Fw109-5) obtained by Matthew Fields' group from the Oak Ridge IFC site soil represents a fourth group in this genus with the ability to reduce metals (M. Fields, personal communication). All *Anaermyxobacter* isolates reduce U(VI) to U(IV) and share other characteristics with strain 2CP-C. Interestingly, at least six of the new *Anaeromyxobacter* isolates reduced U(VI) at different rates suggesting that the Oak Ridge IFC harbors *Anaeromyxobacter* spp. that share similar 16S rRNA gene sequences but exhibit distinct U(VI) reduction capabilities (i.e., rates). Isolate FRC-W, obtained from FRC FW034 (Area 1) soil, showed the highest U(VI) reduction rates and the preliminary characterization of strain FRC-W showed that this organism also reduces 2-CP, fumarate, ferric iron, nitrate, and N_2O with acetate as electron donor.

Iron reduction was observed in microcosms established with Area 1 (FW 034) materials and amended with acetate and ferric citrate at pH 5, 5.5, 6, 6.5, and 7. Ferric iron was reduced in all microcosms including the pH 5 microcosms. Iron reduction activity was lost upon repeated transfers to low pH medium. The resulting enrichment culture fermented citrate to acetate but failed to reduce ferric iron, fumarate and 2-CP. No iron reduction or 2-CP dechlorination was observed after 3 months in microcosms prepared with Area 1 and Area 3 soil samples collected from FB089-01-21 and FWB113-08-01, respectively, at pH 5, 6, and 7 with acetate or 0.1 mM acetate plus hydrogen as electron donors and ferric citrate or 2-CP as electron acceptors.

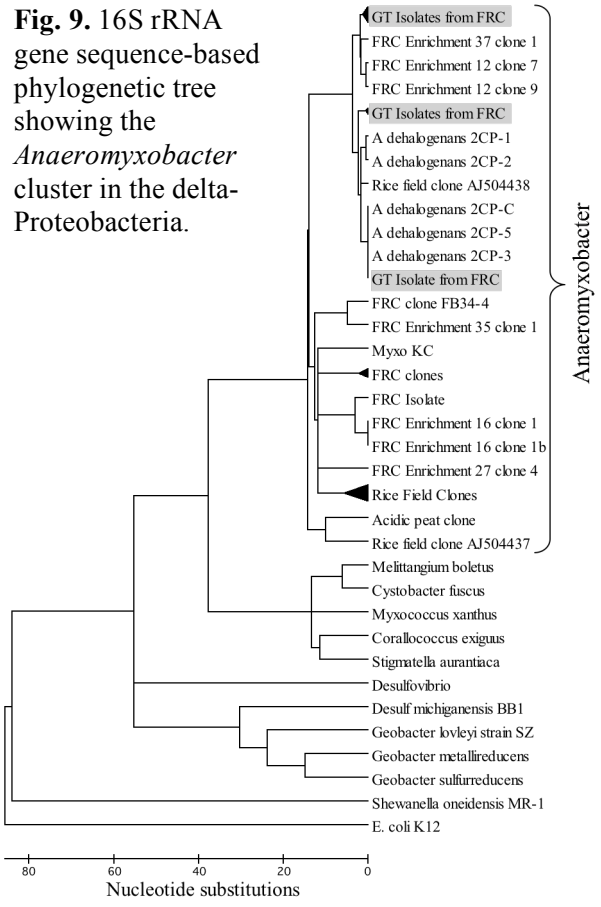


Fig. 9. 16S rRNA gene sequence-based phylogenetic tree showing the *Anaeromyxobacter* cluster in the delta-Proteobacteria.

Objective 4. Design 16S rRNA gene-targeted PCR primers to specifically detect and quantify *Anaeromyxobacter* spp. in pure cultures, consortia, and environmental samples

The goals were to design PCR assays for sensitive and specific detection of *Anaeromyxobacter* 16S rRNA genes and to develop quantitative real time PCR (qPCR) assays for accurate and reproducible

enumeration of *Anaeromyxobacter* cells in laboratory cultures and environmental samples. To accomplish these tasks, available *Anaeromyxobacter* 16S rRNA gene sequences and those of related delta-Proteobacteria (*Myxococcus*, *Stigmatella*, *Desulfitobacterium*, *Geobacter* and *Desulfuromonas*) were obtained from GenBank and aligned using the MegAlign program of the Lasergene software package (DNA Star Inc., Madison, Wisconsin). PCR primer pairs specific for the entire *Anaeromyxobacter* group were designed toward promising regions of the 16S rRNA gene using the Oligo Design and Analysis Tools available online (www.idtdna.com). A forward primer designed by Petrie et al (2003) was used with a newly generated reverse primer for enhanced detection of *Anaeromyxobacter* spp. in soil. Additional primer pairs were designed to specifically detect a variable region of the 16S rRNA gene and identify the four highlighted *Anaeromyxobacter* groups (Fig. 10). Due to subtle differences between *Anaeromyxobacter* isolate and clone sequences, it was impossible to design primers to detect all members of some of the larger branches within the *Anaeromyxobacter* genus.

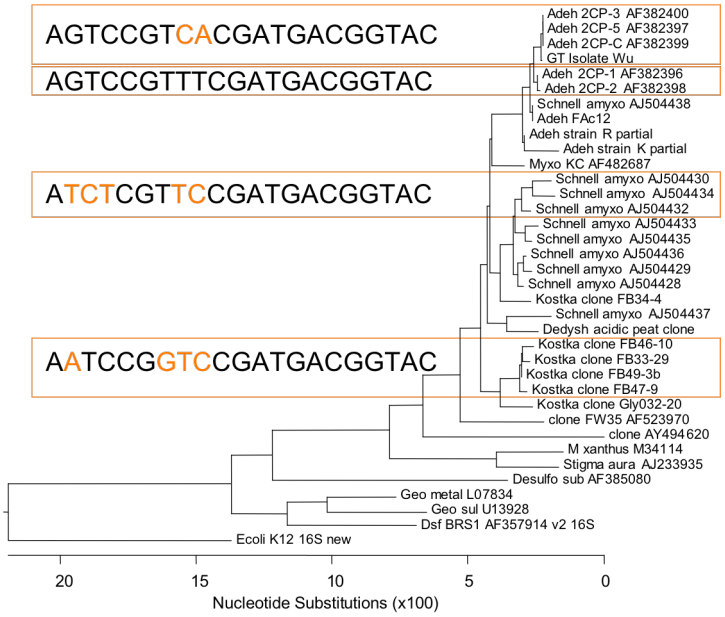


Figure 10. 16S rRNA gene sequence-based phylogenetic tree of *Anaeromyxobacter* shown with the corresponding sequences that were targeted for specific detection of subgroups.

Linear hybridization TaqMan probes and corresponding primers were designed with the Primer Expresss software (Applied Biosystems, Foster City, CA). One primer pair and probe target the *Anaeromyxobacter* genus as a whole, and amplifies a 68 bp fragment. Another set of forward and reverse primers were used with two different probes to distinguish unique regions of the 16S rRNA gene that would delineate two specific *Anaeromyxobacter* subgroups (i.e., 2CP-C-like and 2CP-1-like). Probe sequences were designed to a portion overlapping the variable region highlighted in Figure 10. BLAST analysis of each probe and primer sequence, designed for qualitative and quantitative analysis, suggested that they were specific to the target sequences. To verify that the qualitative primers amplified their target

gene fragment with desired specificity, purified genomic DNA of available *Anaeromyxobacter* isolates and related delta-proteobacteria was subjected to PCR using a mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany), which were tested at annealing temperatures ranging from 54 to 64°C, with the best amplification occurring at 60°C. TaqMan probes and primers were employed at the temperatures recommended by the Primer Express program, and operated in the standard ABI 7500 mode (Applied Biosystems, Foster City, CA). Figure 11 shows an example of the 10-fold diluted genomic DNA of *A. dehalogenans* strain 2CP-1 and qPCR with the Adehal_total, the 2CP-1 and the 2CP-C probes. As expected, amplification occurred with the Adehal_total and the 2CP-1 probes but not with the 2CP-C probe. The three TaqMan qPCR probes were synthesized by Applied Biosystems and carried different fluorophores to facilitate future multiplex PCR applications for simultaneously detecting total *Anaeromyxobacter* (VIC fluorophore) as well as the 2CP-C (NED fluorophore) and 2CP-1 (FAM fluorophore) subgroups (in terms of labor, time and costs).

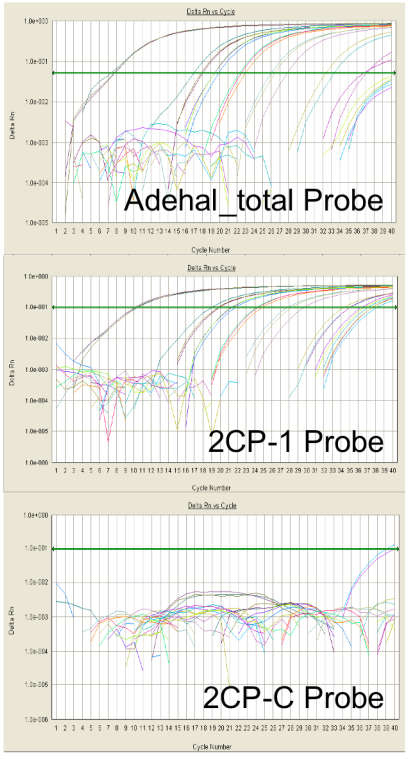


Figure 11: Serial 10-fold dilutions of strain 2CP-1 genomic DNA analyzed with the three TaqMan probe-primer sets gave similar quantification results with the general *Anaeromyxobacter* and 2CP-1 sets but no amplification occurred with the 2CP-C set.

These results demonstrate that qPCR with Taqman linear hybridization probe detection chemistry distinguishes the 2CP-C and 2CP-1 *Anaeromyxobacter* subgroups. Further probe refinement and the application of molecular beacon detection chemistry, which has higher discrimination power (i.e., sequences with 1 bp difference can be detected), will allow discrimination of *Anaeromyxobacter* strains with highly similar 16S rRNA gene sequences but different physiological properties. For example, the new Oak Ridge IFC site isolates are 99% similar at the 16S rRNA gene level but reduce U(VI) at different rates. In addition, the possibility of multiplex PCR, which allows the simultaneous quantification of up to four targets in the same PCR tube, will make the analysis more efficient.

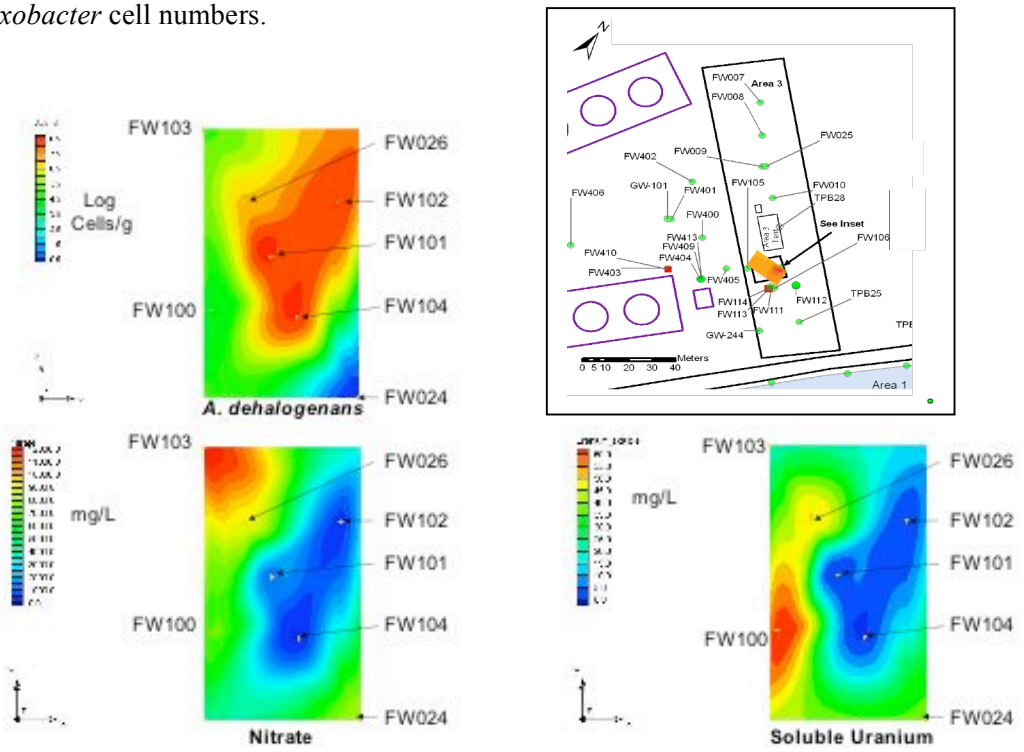
Objective 5. Assess *Anaeromyxobacter* spp. distribution and abundance at the Oak Ridge IFC site.

To date, we have designed 16S rRNA gene-targeted qPCR assays targeting the entire *Anaeromyxobacter* group and specific for two distinct *Anaeromyxobacter* subgroups, the 2CP-C and the 2CP-1

clusters. The quantitative tools have been successfully applied to defined laboratory cultures and are currently being refined for application to environmental samples.

The qPCR approach targeting the entire *Anaeromyxobacter* group (i.e., Adehal_total Probe) was applied to DNA samples collected from multiple Area 3 wells at the Oak Ridge IFC site. These samples were kindly provided by T. Gentry and J. Zhou. These analyses corroborated that *Anaeromyxobacter* spp. are present at the Oak Ridge IFC site though the organisms are not evenly distributed in the subsurface. Most interestingly, *Anaeromyxobacter* spp. 16S rRNA genes increased in abundance at two locations in Areas 1 and 3 following biostimulation with ethanol (Wu et al. 2006a,b). No increase in *Anaeromyxobacter* spp. 16S rRNA genes occurred in nearby locations that were not impacted by biostimulation. Figure 11 shows contour plots generated with the GMS Groundwater Modeling System software package of nitrate concentrations, U(VI) concentrations and *Anaeromyxobacter* spp. abundance in Area 3 following ethanol biostimulation. The contour plots show that enhanced nitrate removal and U(VI) reduction correlate with increased abundance of *Anaeromyxobacter* spp. suggesting that *Anaeromyxobacter* spp. are involved in U(VI) reduction and/or nitrate removal.

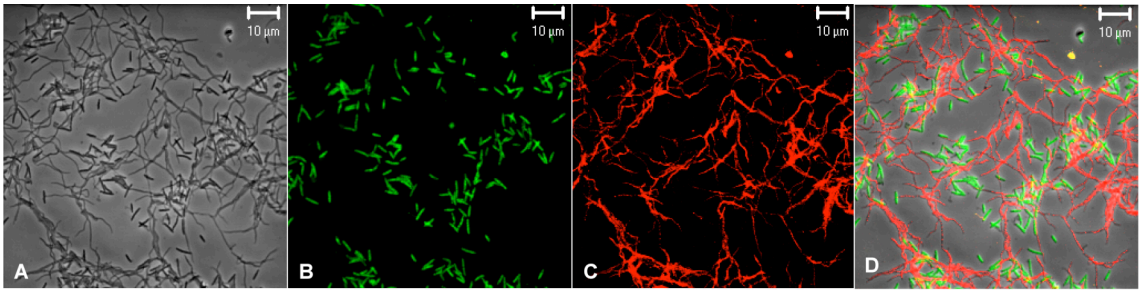
Figure 11. Contour plots of nitrate concentrations, U(VI) concentrations and abundance of *Anaeromyxobacter* spp. at the Criddle/Jardine pilot test plot in Area 3 at the Oak Ridge IFC site. The inset on the top right depicts the location of the test plot in Area 3. Blue indicates low concentrations of nitrate and U(VI) and low *Anaeromyxobacter* cell numbers; red indicates high concentrations of nitrate and U(VI) and high *Anaeromyxobacter* cell numbers.



These findings strongly suggest that *Anaeromyxobacter* spp. respond to biostimulation and contribute to U(VI) reduction and/or nitrate removal. Thus, enhanced rates of contaminant removal can be expected following enhanced treatment (i.e., biostimulation) at the Oak Ridge IFC site.

A promising tool for visualizing cells of interest in their native environment is Fluorescence *In Situ* Hybridization (FISH). With FISH, target cell distribution patterns, dynamics, growth behavior, and co-localization with other microbes (i.e., juxtapositioning) can be studied under *in situ* conditions. FISH has become a standard tool in microbial ecology but has seen limited use to explore metal-reducing bacteria contributing to U(VI) reduction in subsurface environments. We started collaborating with Dr. Natuschka Lee from the Technical University Munich to apply FISH technology to *Anaeromyxobacter* spp. and *Geobacter* spp. Dr. Lee is an expert in applying different FISH approaches for visualizing subsurface bacteria including standard 16S rRNA-targeted FISH and advanced FISH procedures that target functional genes (i.e., RING-FISH, *R*ecognition of *IN*dividual *G*enes). Dr. Loeffler was invited to give two presentations at an international course on FISH and stable isotope probing (SIP) in December 2006 in Munich. He participated in this course and subsequently conducted exploratory FISH experiments in collaboration with Dr Lee. Figure 12 shows FISH detection of *Anaeromyxobacter dehalogenans* strain 2CP-C and its close relative *Myxococcous xanthus*.

Figure 12. FISH of an *Anaeromyxobacter dehalogenans* and *Myxococcous xanthus* co-culture with group-specific probes.



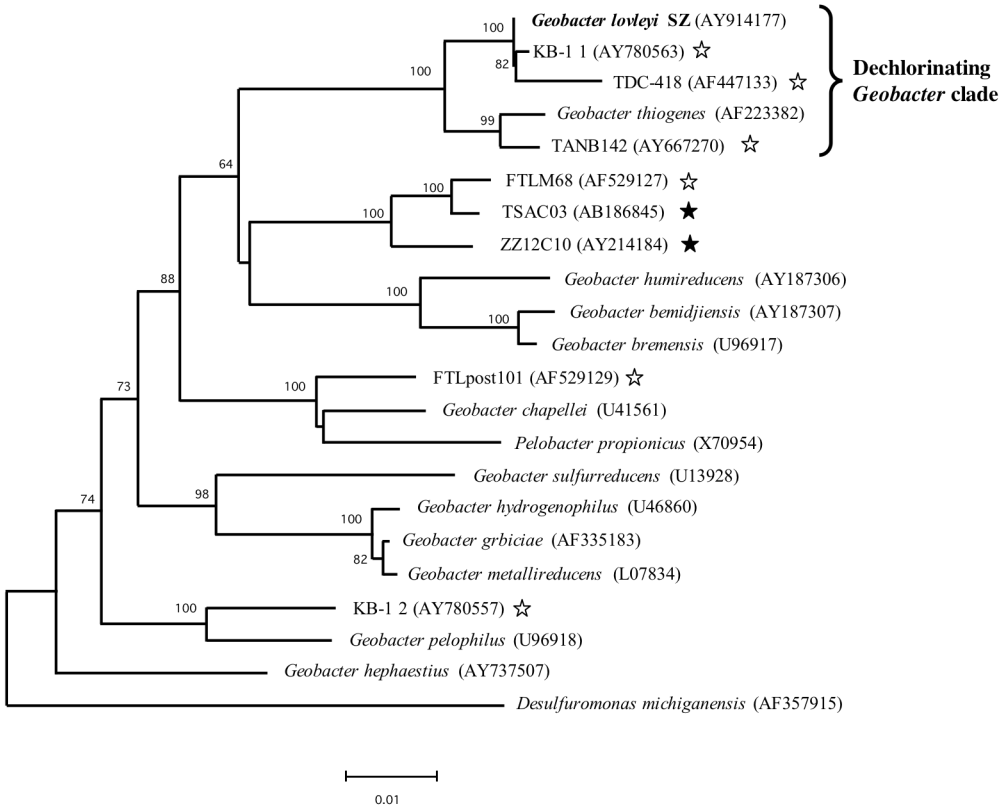
A: Phase contrast **B:** *Myxococcus* probe (green) **C:** *Anaeromyxobacter* probe (red) **D:** A+B+C

The FISH approach clearly distinguishes *Anaeromyxobacter dehalogenans* from *Myxococcous xanthus*, both common soil bacteria. These preliminary results are encouraging and we are excited to continue these efforts and develop FISH-based tools for the specific visualization and activity monitoring of *Anaeromyxobacter* spp. at uranium-contaminated DOE sites.

Objective 6. Explore the contributions of *Geobacter lovleyi* to contaminant detoxification at the Oak Ridge IFC site

The tetrachloroethene and U(VI)-reducing isolate SZ was described as the type strain of a new species, *Geobacter lovleyi* (Sung et al. 2006). Together with *Geobacter thiogenes* and several environmental clone sequences, *Geobacter lovleyi* strain SZ belongs to a dechlorinating clade within the *Geobacteraceae* (Fig. 13).

Figure 13. Inferred phylogenetic tree of strain SZ and related species and environmental clones based on 16S rRNA gene sequences. The bootstrap values at the nodes are based on 1,000 iterations, and only values >50% are shown. The tree was generated for 1,290 bp of aligned positions using Clustal W (MegAlign). Stars indicate environmental clone sequences; open stars indicate that these sequences were retrieved from dechlorinating enrichments cultures or chloroethene-contaminated sites. The scale bar represents 1 bp substitution per 100 nucleotides.



The discovery of a tetrachloroethene-dechlorinating *Geobacter* species is relevant because many U(VI)-impacted DOE sites are also contaminated with chlorinated ethenes.

To evaluate the distribution of *Geobacter lovleyi* strain SZ in environmental samples, we designed direct and nested PCR assays using 16S rRNA gene-targeted primer pairs that distinguished strain SZ from other known chlorinated ethene-dechlorinating bacteria and closely related *Geobacter* isolates, including its closest cultured relative, *G. thiogenes*. Detection limits for direct and nested PCR were approximately 1 x 10^6 and 1 x 10^4 16S rRNA gene copies per ml of template DNA, respectively. A qPCR approach increased the sensitivity to as few as 30 16S rRNA gene copies per ml of template DNA but was less specific. Melting curve analysis and comparison of the shapes of amplification plots identified false positive signals and distinguished strain SZ from *G. thiogenes* in qPCR analysis. These new detection and quantification tools detected strain SZ-like amplicons in PCE-dechlorinating consortia, including the bioaugmentation consortium KB-1, and two chlorinated ethene-impacted groundwater samples.

Strain SZ-like organisms were detected in community genomic DNA extracted from 13 of 22 Oak Ridge IFC site groundwater samples. As shown in Figure 14A, specific amplicons were obtained with direct and nested PCR from samples of wells FW026, FW101-2, and FW102-3 located within the Area 3 biostimulated zone (Wu et al. 2006a,b). Nested PCR yielded an additional positive signal with DNA collected from well FW029 (Area 1), which was also influenced by biostimulation (Fig. 14A) (Istok et al. 2004). Sequence analysis of at least 350 bp of all amplicons confirmed sequence identity with the 16S rRNA gene sequence of strain SZ. No amplification was observed through direct or nested PCR (Fig. 14A) with samples from wells FW016 (Area 1), TPB16 (Area 2), and FW106 (Area 3), which are located in areas that received no biostimulation treatment. The geochemistry (e.g., low pH and high nitrate) of well FW106 is similar to that of the Area 3 biostimulated zone prior to conditioning and periodic ethanol amendment (Wu et al. 2006a,b). Detection of strain SZ-like organisms in samples from the biostimulated plot, but not from well FW106, indicates that strain SZ responded to biostimulation at the Oak Ridge IFC.

As depicted in Figure 14B, strain SZ-like organisms could also be quantitatively detected in various groundwater samples collected within the uranium bioreduction test plot at the Oak Ridge IFC site (Wu et al. 2006a,b). In February 2004, approximately one month after the initial amendment of ethanol to the inner recirculation loop (see Materials and Methods for a brief description of the inner and outer recirculation loops), the number of strain SZ cells was less than 5 x 10^4 per liter groundwater (Fig. 14B). Minimal amplification (i.e., fluorescence) was observed in most of the samples from February 2004 (see asterisks [$*$] in Fig. 14B), and approximate cell number estimates for these samples were extrapolated from the linear qPCR standard curve. The melting curves for these samples did not have the characteristic strain SZ peak, but instead several smaller peaks were visible (Fig. 14C). All samples that could be accurately quantified had melting curves with the characteristic peak for strain SZ (Fig. 14C). These results demonstrate that melting curve analysis was useful to distinguish nonspecific amplification and identify false positive signals with environmental samples. Samples taken following biostimulation

indicated substantial growth of strain SZ-like organisms (Fig. 14B). For example, cell numbers in samples from the inner recirculation loop injection well (FW104) increased by over three orders-of-magnitude from February 2004 to May and August 2005 (Fig. 14B). The samples from May and August 2005 were taken approximately 1-1.5 years after the onset of periodic ethanol addition to the inner recirculation loop, indicating that strain SZ persisted at high levels in the biostimulated zone throughout the biostimulation period. In August 2005, strain SZ cell numbers ranged from 10^5 - 10^6 per liter groundwater in regions impacted by biostimulation (Fig. 14B). In contrast, the number of SZ cells was significantly lower in samples taken at the same time from aquifer regions not impacted by biostimulation (Fig. 14B).

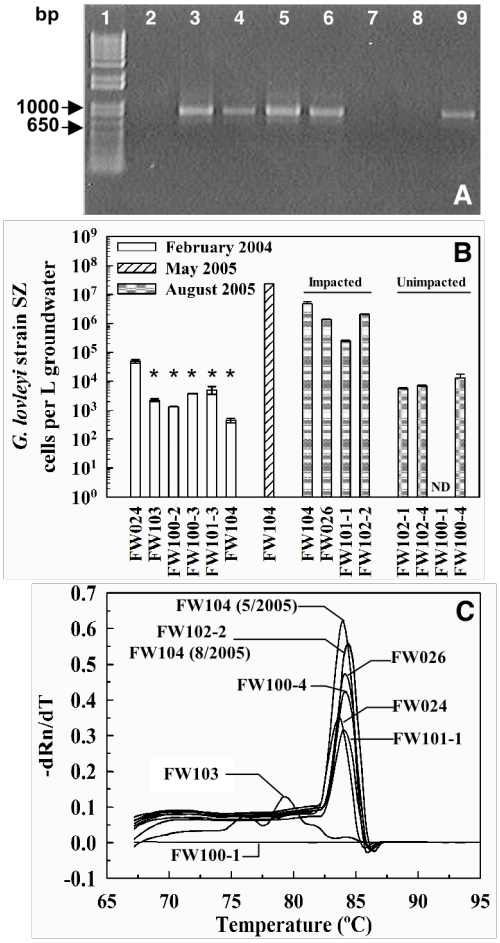


Figure. 14. (A) Detection of strain SZ-like organisms via nested PCR in uranium-contaminated Oak Ridge IFC site groundwater taken from regions impacted by biostimulation (wells FW026 [lane 3], FW029 [lane 4], FW101-2 [lane 5], FW102-3 [lane 6]) or unimpacted by biostimulation (wells FW016 [lane 2], FW106 [lane 7], and TPB16 [lane 8]). Lane 1 is the DNA size marker (Invitrogen), and lane 9 is genomic DNA of strain SZ. (B) Average *G. lovleyi* strain SZ cell numbers per liter of Oak Ridge IFC site groundwater in a pilot-scale uranium bioreduction demonstration plot. Samples were taken approximately 1 month after the initial ethanol amendment (February 2004) and in May and August 2005, after 1-1.5 years of periodic ethanol biostimulation. The samples from August 2005 came from aquifer regions impacted or not impacted by biostimulation, as indicated. Error bars indicate standard deviations of triplicate qPCR reactions and are not shown when they are too small to depict. The asterisks (∗) indicate that the cell number estimates were extrapolated values outside the range of accurate quantification and that the melting curves did not contain the characteristic peak for strain SZ. ND; fluorescence was not detected. (C) Melting curves following qPCR for representative samples from the Oak Ridge IFC site. The melting curves represent averages of triplicate qPCR reactions for each sample. -dRn/dT represents the negative derivate of the reported fluorescent signal (Rn) with respect to temperature (T). The T_m for the target amplicon is at the maximum rate of change (i.e., the greatest -dRn/dT) for each melting curve.

Our observations indicate that strain SZ-like organisms were present at the Oak Ridge IFC site and, importantly, responded to biostimulation. The addition of ethanol and/or adjustment of other geochemical parameters (e.g., pH, nitrate removal (Wu et al. 2006a,b)) lead to subsequent growth of strain SZ-like organisms. Although strain SZ cannot utilize ethanol as a direct electron donor, ethanol fermentation likely produced hydrogen and acetate, electron donors used by strain SZ (Sung et al. 2006). Acetate was detected in portions of the biostimulated zone following ethanol addition (Wu et al. 2006b). Since strain SZ respires U(VI) (Sung et al. 2006, Sanford et al. 2007), detection of strain SZ-like organisms after biostimulation suggests their involvement with U(VI) bioreduction at the Oak Ridge IFC site. Previous work has demonstrated that *G. lovleyi* strain SZ reduces U(VI) and PCE simultaneously (Sung et al. 2006), and hence, strain SZ is a promising candidate for *in situ* bioremediation at the mixed waste Oak Ridge IFC site (US DOE 1999, Wu et al. 2006a). The identification of key organisms contributing to contaminant detoxification (e.g., U(VI) reduction and reductive dechlorination) represents a targeted approach for bioremediation monitoring. Although comprehensive information of the microbial community composition and dynamics at bioremediation sites is desirable, the application of targeted monitoring approaches to delineate and quantify the contributions of select organisms may produce tangible results in the short term. Monitoring key contributors such as strain SZ-like organisms and other U(VI)-reducing bacteria (e.g., *Anaeromyxobacter* spp. and other metal reducers) to U(VI) bioreduction at the Oak Ridge IFC site and other uranium-impacted sites may provide sufficient information for productive implementation of bioremediation technologies.

Conclusions

1. *Anaeromyxobacter* spp. are versatile, facultative microaerophiles that utilize U(VI) as growth-supporting electron acceptor.
2. *Anaeromyxobacter dehalogenans* strain 2CP-C requires hydrogen as electron donor for U(VI) reduction under growth condition.
3. *Anaeromyxobacter dehalogenans*, *Geobacter lovleyi* and *Geobacter sulfurreducens* respire U(VI) but produce lower than expected biomass yields with U(VI) as electron acceptor.
4. *Anaeromyxobacter dehalogenans* reduces U(VI) in the presence of competing electron acceptors (e.g., ferric iron, 2-chlorophenol, N_2O, fumarate). U(VI) reduction occurs in the presence of HFO but at reduced rates. The addition of nitrate to U(VI)-reducing cultures resulted in U(IV) reoxidation, but U(VI) reduction resumed following the consumption of nitrite.
5. *Anaeromyxobacter* spp. use N_2O as metabolic electron acceptor. N_2O is a strong oxidant but does not cause U(IV) reoxidation or affect U(VI) metabolism in *Anaeromyxobacter* spp.

6. PCR-based approaches accurately and reproducibly detect *Anaeromyxobacter* spp. 16S rRNA gene sequences in environmental samples.
7. *Anaeromyxobacter* spp. are present at the Oak Ridge IFC site and respond to biostimulation with ethanol.
8. Several *Anaeromyxobacter* isolates were obtained from Oak Ridge IFC site materials.
9. All *Anaeromyxobacter* isolates reduce U(VI) but the rates of U(VI) reductions differ between strains.
10. Members of the dechlorinating *Geobacter* clade (i.e., *Geobacter lovleyi*-type organisms) were detected at the Oak Ridge IFC site. *Geobacter lovleyi*-type organisms increased in abundance following ethanol biostimulation.
11. *Anaeromyxobacter* spp. (and *Geobacter* spp.) are present in DOE contaminated subsurface environments. *Anaeromyxobacter* spp. and members of the dechlorinating *Geobacter* clade native to the Oak Ridge IFC site respond to biostimulation and are likely relevant contributors to U(VI) immobilization.
12. Further exploration of the contributions of dissimilatory metal-reducing bacteria (i.e., *Anaeromyxobacter* spp.) to U(VI) reduction and immobilization is warranted. In particular, new and improved tools for site assessment and process monitoring are needed. The integrated application of new approaches will significantly contribute to moving bioremediation from a relatively empirical practice to a predictable science with widespread application at contaminated DOE sites.

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