

INVESTIGATING IN SITU BIOREMEDIATION APPROACHES FOR
SUSTAINED URANIUM IMMOBILIZATION INDEPENDENT OF NITRATE REDUCTION

Final Technical Report

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**FINAL TECHNICAL REPORT
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1. Introduction: Project Goal and Background Information

The primary goal of this project was to gain a better understanding of the microbiological diversity and community composition in low-pH, high-nitrate environments. The research made use of enrichment cultures grown from DOE Oak Ridge Field Research Center (FRC) sediments in T.J. Phelps' lab at Oak Ridge National Laboratory (ORNL), with the intention of identifying those bacterial species capable of reducing, and therefore immobilizing, metals and radionuclides without the use of nitrate/nitrite and without requiring the removal of nitrate/nitrite.

Previous studies have shown that microbial diversity is high in sediments contaminated with radionuclides and metals (Anderson et al., 2003; Chang et al., 2001; Nevin et al., 2003; Holmes et al., 2002; Petrie et al., 2003) and that low G+C Gram-positive species of bacteria were present in enrichments from low-pH, high-nitrate contaminated sediments (Petrie et al., 2003). This was particularly important for the current project, as it was hypothesized that under strictly anaerobic conditions certain Gram-positive genera, such as *Clostridium*, may carry out uranium reduction in low pH environments without the need or ability to use nitrate/nitrite as an electron acceptor. Chemically, a low-pH environment has the potential to inhibit metal reducers from utilizing reduced U(IV) as an electron donor for nitrate reduction (Finneran et al., 2002), leading to a stable U(IV) species in low-pH, high-nitrate sediments. Uranium stability under these conditions provides key knowledge required to develop new protocols that will aid in the successful bioremediation of DOE waste sites.

2. Methods Development

For this project, it was necessary to devote a considerable amount of time to the development and optimization of the specialized methods for analysis of microbial communities in the enrichment cultures from FRC sediments that were grown in T. J. Phelps' lab at ORNL. The methodologies found to be most useful are detailed below.

2.1. PCR Analysis of 16S rRNA Gene Sequences

Microbial community DNA was extracted directly from filters using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, Calif.). The manufacturer's instructions were modified to increase DNA yields from filters. Filters were cut in half. The first half was cut into small pieces and transferred to bead-beating tubes. The second filter half was immediately stored at -20°C. Initial bead beating times for each filter half were increased to 30 min, and alternating heating and bead beating times for the cell lysis step were increased to 45 min each. Buffer volumes were also slightly increased throughout the protocol. Extracted bacterial community DNA was eluted in 50 µl 10 mM Tris-HCl.

Bacterial 16 rRNA genes were amplified from community DNA by PCR, using primers B27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and B907R (5'-CCG TCA ATT CMT TTR AGT TT-3'). PCR mixtures of 150 µl contained 2.5 µM of each primer, 3.5 units of Takara LA

Taq, 15 µl of 10X LA PCR Buffer with 25 mM Mg²⁺, and 2.5 mM dNTP mix (Takara Biomedicals). Prior to the beginning of the cycle, 20 ng of DNA was added and the reaction was split into three tubes with 50 µl each. PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems) with the following conditions: initial DNA denature of 3 min at 96°C, followed by 30 cycles of 30 sec at 96°C, 45 sec at 56°C, 1 min at 72°C, and then a final extension of 5 min at 72°C. PCR products of approximately 900 bp were cloned with a Topo TA cloning kit (Invitrogen, Carlsbad, Calif.) and libraries of approximately 50 transformants were made for each sample.

Plasmid inserts were screened by PCR directly from individual colonies with the M13F and M13R primers and restriction enzyme digest with Hae III at 37°C for 4 h, followed by denaturing of the enzyme at 80°C for 20 minutes. Restriction digests of plasmid inserts were run on 3% gels using a non tris-based buffer, SB buffer (Faster Better Media, LLC, Hunt Valley, Md.), which allows the high percentage gels to be run at a higher voltage without spending hours in a cold room. Restriction digest gels were run at 275 V and approximately 27°C. Clone inserts with unique restriction patterns were cleaned with QIAquick PCR purification columns (QIAGEN, Valencia, Calif.) and sequenced using primer G (5'-CCA GGG TAT CTA ATC CTG TT-3') at the Florida State University DNA sequencing facility. Sequences were aligned against close relatives from the Ribosomal Database Project (RDP) using the GDE (version 1.7.5) software package and dendrograms were constructed using PAUP* 4.0 (Sinauer Associates, Sunderland, Mass.). Freezedowns of all sequenced clones retrieved during this analysis have been stored in 8% DMSO at -80°C.

2.2. T-RFLP Analysis of Bacterial Community Structure

T-RFLP analysis of the bacterial community structure was based on amplified 16S rRNA genes. Triplicate analyses using variable DNA concentrations were performed for each sample in order to better define bacterial diversity through comparisons with clone libraries.

PCR amplification of approximately 900 bp of the 16S rRNA gene was accomplished using the same primer set as previously described above for cloning and sequencing, except that the forward primer contained the blue fluorescent tag 6-FAM (Applied Biosystems) for identification of terminal fragments. Restriction digests of tagged amplicons were run for 4 hours at 37°C using HaeIII and MnlI enzymes (New England Biolabs, MA) separately. T-RFLP analysis of individual clones representing the different groups was performed to confirm community peak identifications.

Reactions for T-RFLP analysis were prepared in triplicate for each sample, with 4 µl, 2 µl, and 1 µl of digested tagged amplicons added (~400 ng, 200 ng, and 100 ng, respectively). The GeneScan 500 ROX internal size standard (Applied Biosystems, UK) was also included at a concentration of 4 fmol per reaction (0.5 µl). Each reaction was combined with 12 µl of deionized formamide and sent to the Florida State University DNA Sequencing Laboratory for analysis. T-RFLP reactions were run on a 3130xl Genetic Analyzer (Applied Biosystems, Inc.) using POP 7 as the polymer, and 16 capillaries for processing samples.

3. Principal Results and Findings

3.1. Enrichment Culture Studies

The enrichment culture studies were carried out in T. J. Phelps' laboratory at ORNL, but the results are included here to provide a context for the analytical results obtained in the PI's laboratory at Florida State.

Transfers from enrichment cultures containing uranium-contaminated sediments from the Oak Ridge FRC site demonstrated the capacity for reduction of uranium in the presence of nitrate (Table 1). Successful enrichments from U-contaminated sediments demonstrated nearly complete loss of uranium from solution, presumably via biologically mediated reduction (~90% reduction at ~10 ppm starting concentration) with less than 10% loss of ~850 ppm nitrate (Table 1) from pH 4.9-5.6, with 10-30 mM methanol or glycerol as the energy source. Higher-pH enrichments also demonstrated similar U reduction capacity (data not shown) with 5-30% nitrate loss within one week, although the timescale for uranium reduction varied widely with different cultures and enrichments.

Table 1.

Sample	Electron donor	Buffer pH	% nitrate loss	% U loss
1	methanol	6.2	18.8	98.9
2	methanol	6.2	13.7	99.0
3	glycerol	6.2	7.4	92.9
4	methanol	5.9	8.2	98.4
5	methanol	5.9	4.6	90.1
6	glycerol	5.9	7.6	91.8
7	methanol	5.7	11.9	98.8
8	methanol	5.7	22.1	98.2

Observed uranium loss from solution (Table 1) was verified to occur through reduction by use of an extraction procedure followed by Kinetic Phosphorescence Analysis (KPA). In KPA, the fluorescence signal results only from U(VI) species and is not sensitive to U(IV). Differences between concentrated nitric acid-extracted uranium and carbonate-extracted uranium represent the reduced uranium pool (Fig. 1). Concentrated nitric acid extraction with exposure to air recovers the total uranium, while carbonate solubilizes much of the sorbed and precipitated U(VI). The very small difference between aqueous and carbonate-extracted U(VI) indicated that the majority of uranium was likely reduced (Fig. 1).

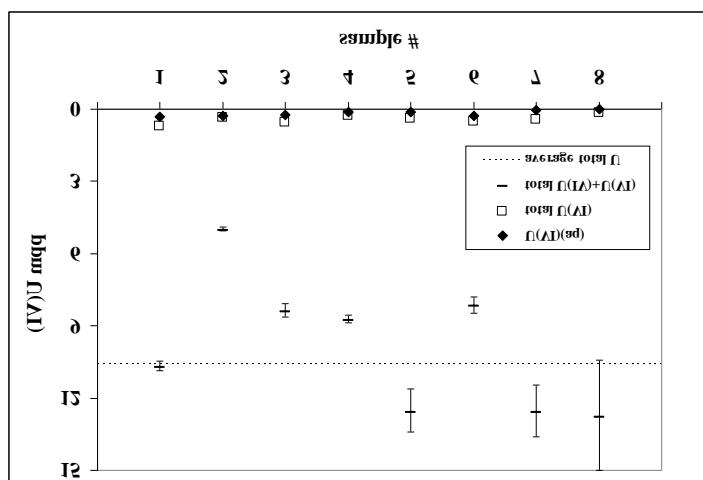


Figure 1. U(VI) concentrations measured in enrichment cultures using various chemical extractions. Sample numbers correspond with Table 1.

Additional experiments were carried out to determine if uranium would be chemically reduced in equivalent medium containing an electron donor but with a chemical reductant instead of an inoculant. 0.025% cysteine HCl alone did not cause loss of U(VI) in treatments with or without 850 ppm nitrate (Fig. 2), and no significant reduction of uranium was observed after 40 days. Thus, the reduction of uranium in enrichment cultures depended upon biological activity and required coupled biogeochemical processes. The kinetics of uranium reduction (Fig. 3) did not support a direct coupling between reduction processes and cell growth. The observed turbidity of cell suspensions suggested that while reduction occurred over the time scale of tens of days (Fig. 3), maximum growth occurred within ~5 days.

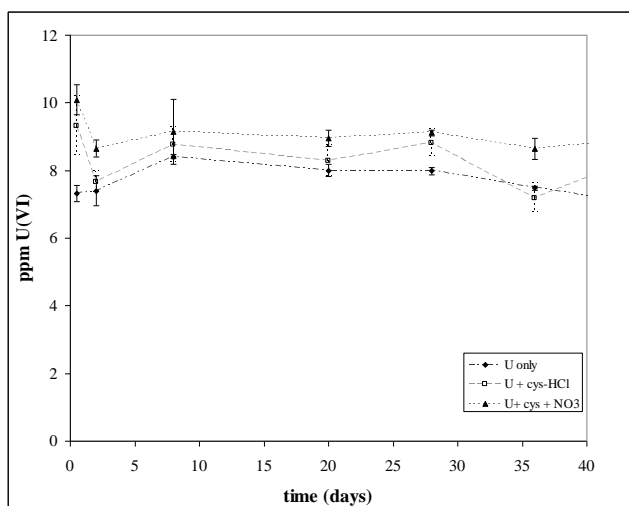


Figure 2. Control replicates in basal medium with and without reducing agent and nitrate.

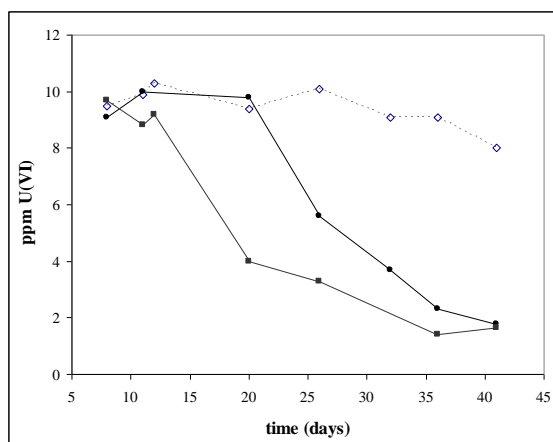


Figure 3. Time dependence of aqueous U concentrations in representative transfers (closed symbols) and uninoculated control (open symbols). Tubes contained no cysteine-HCl.

3.2. 16S rRNA Gene Sequence Analyses

As noted above, bacterial communities in the aforementioned enrichment cultures were analyzed in the PI's laboratory at Florida State. The most significant results of these analyses are summarized in this and the next section.

Cloning and sequencing of 16S rRNA genes indicate that four principal groups of bacteria were present in the enrichments. The largest and seemingly most diverse group consisted of low G+C Gram-positive bacteria (Cluster A; Figure 4) such as *Clostridium* (Cluster A, iv; Fig. 4) and Clostridia-like organisms, including members of the: 1) Lachnospiraceae (Cluster A, i), 2) Peptostreptococcaceae (Cluster A, ii), 3) Eubacteriaceae (Cluster A, iii), and 4) Acidaminococcaceae (Cluster A, v). Sequence similarities for this large group of clone sequences ranged from 88% to 99%, including individual clones most closely related to *Clostridium* sp., *Sedimentibacter* sp., and *Desulfotomaculum* sp.

A separate sequence cluster (Cluster B, Fig. 4) contained a single clone most closely related to *Paenibacillus graminis* (sequence similarity ~97%), a Gram-positive bacillus. Cluster C was composed of Gamma Proteobacteria that were most closely related to the genus *Aeromonas*, with sequence similarities ranging from 94% to 99%. Additionally, a clone sequence ~98 % identical to *Shewanella putrefaciens* was recovered. Cluster D contained clone sequences that fell within the phylum Bacteroidetes. Most of the clone sequences within this group were most closely related to unknown or uncultured *Bacteroidetes* sp., with sequence similarities between 82% and 99%.

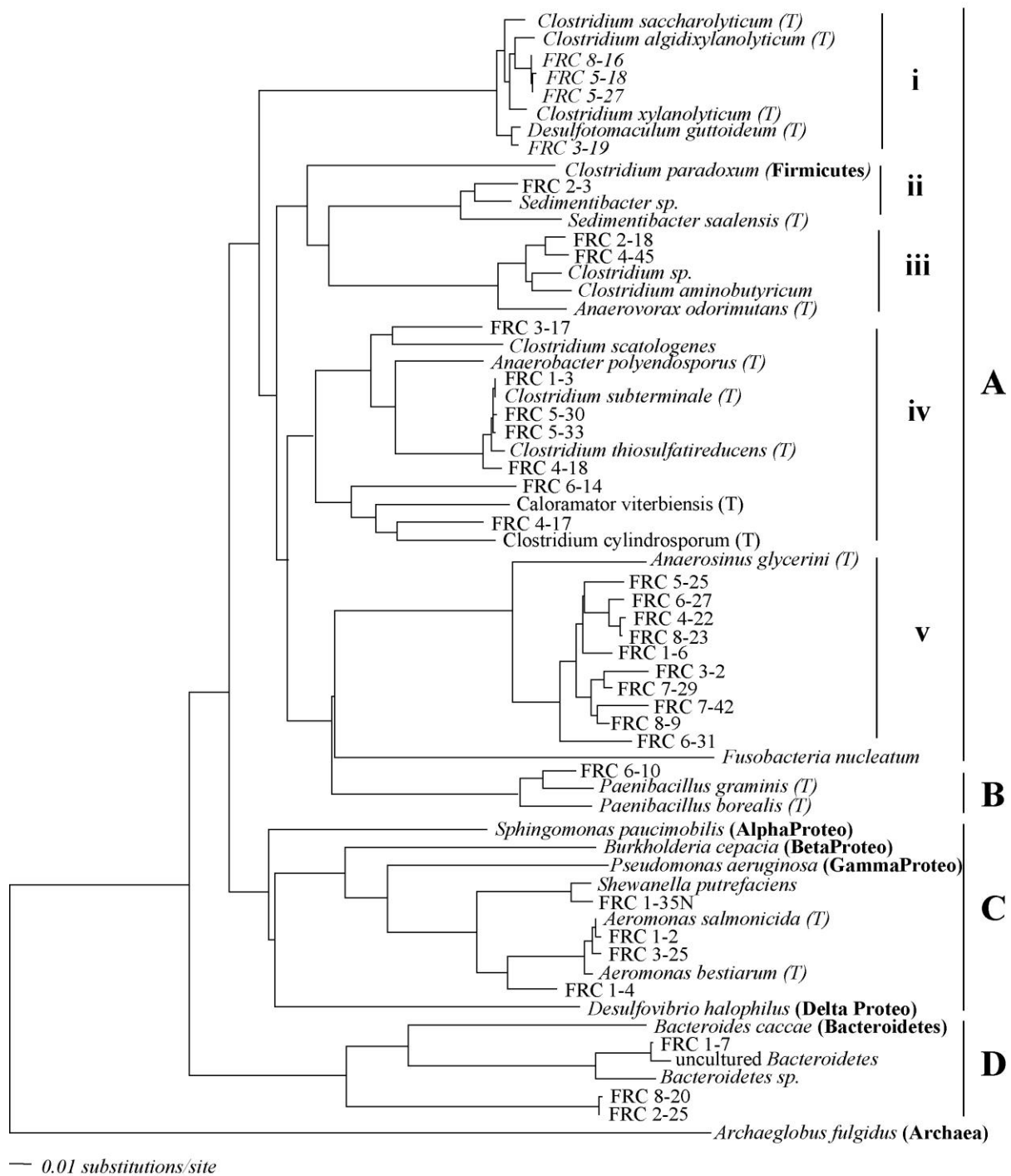


Figure 4. Phylogenetic analysis of bacterial clones from enrichment cultures based on 16S rRNA sequences. Clone IDs correspond to the Table 1 enrichment sample numbers and individual clone IDs. (i.e. 2-3).

3.3. T-RFLP Profile Analysis of Bacterial Community Structure

A larger percentage of the terminal fragment peaks from HaeIII digests were identified (90%) as compared to those samples digested with MnlI (52%), indicating that HaeIII was better suited for the analysis of the types of microbial communities found in these samples. Therefore, only HaeIII profiles are presented here (Fig. 5).

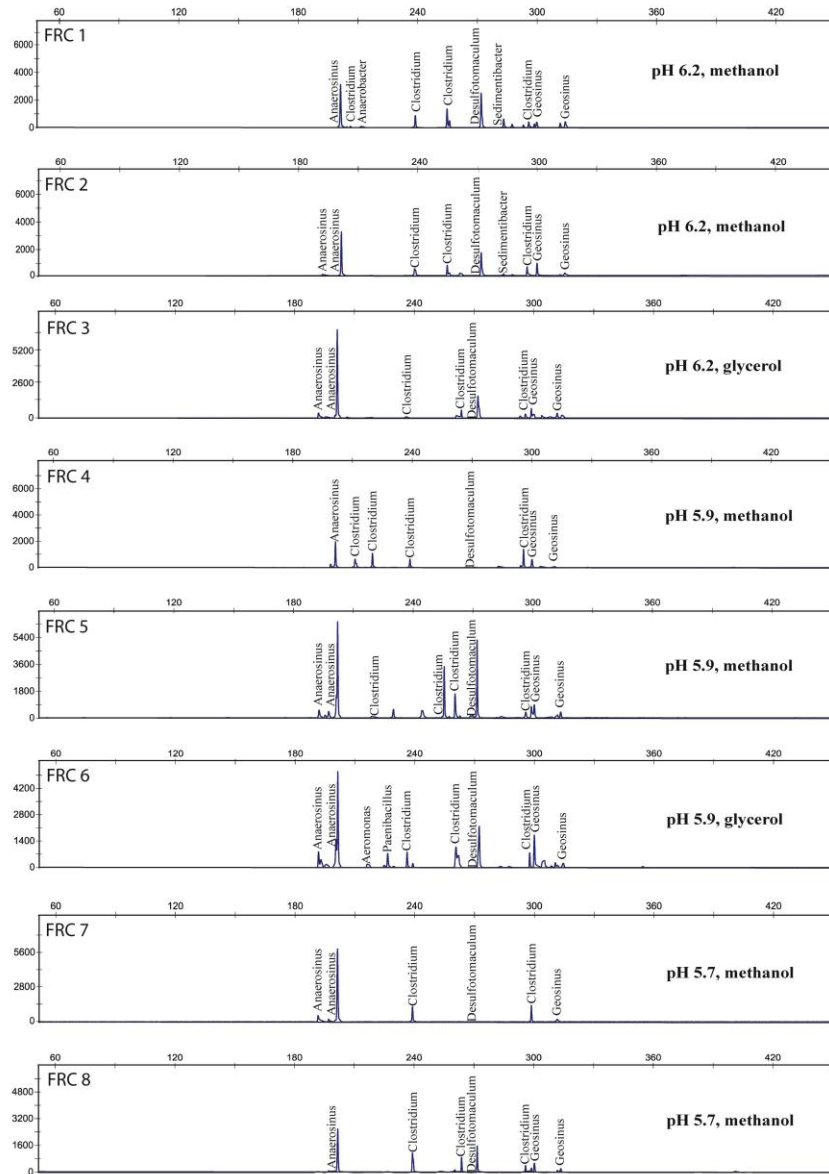


Figure 5. T-RFLP profiles of microbial communities from enrichments corresponding to Fig. 1 and Table 1.

Terminal fragment analysis of 16S rRNA clone sequences (Fig. 5) indicated that the two most dominant groups of organisms present in these enrichments were those most closely related to *Anaerobaculum glycerini* and *Desulfotomaculum guttoideum*, strictly anaerobic members of the phylum Firmicutes. Overall, a considerable amount of diversity was detected among the genera related to *Clostridium*. The aerobic Gamma Proteobacteria and metabolically versatile Bacteroidetes only comprised a very small portion of the bacterial community in all enrichment samples. Those organisms that were most closely related to *Paenibacillus graminis* (similarity rank = 0.85) were present only in enrichment sample FRC 6, which had a pH=5.9, with glycerol as the electron donor. *Sedimentibacter* sp. were only found in samples FRC 1 and FRC 2, where the pH was 6.2 and methanol was the electron donor. Lower pH enrichments with methanol as the electron donor tended to yield overall lower diversity.

4. Conclusions

The research described in this report investigated innovative potential biotransformation strategies to provide long-term stability of immobilized metals and radionuclides without requiring complete nitrate removal in low-pH environments. Enrichments from uranium-contaminated Oak Ridge Field Research Center (FRC) sediments demonstrated the nearly complete reduction of uranium with very little loss of nitrate from pH 4.9-6. A majority of clone sequences retrieved from enrichment cultures were related to *Clostridium* and *Clostridium*-like organisms. During biostimulation, uranium reduction without complete and consistent nitrate removal likely proceeds through coupled biogeochemical processes. Although the enrichment strategy did not target known metal reducers or nitrate reducers, uranium was reduced despite nitrate persistence.

5. Additional Research

During the no-cost extension period of the project, an opportunity arose to carry out a brief collaboration with J. K. Fredrickson at Pacific Northwest National Laboratory (PNNL). The research carried out in the PI's laboratory at Florida State involved phylogenetic characterization of desiccation-resistant bacteria from arid soils at the DOE Hanford Site. Most of these bacteria were found to be members of the genus *Deinococcus*, but representatives of *Chelatococcus*, *Methylobacterium*, and other genera were also detected. The phylogenetic analyses became part of a paper published in *ISME Journal* that linked desiccation resistance with protein oxidation. It was also discovered that several of the *Deinococcus* isolates represent novel species of this genus, and a manuscript describing the new species is now in preparation.

The doctoral student who carried out part of the primary research funded by the grant for which this report is being submitted and all of the collaborative research with J.K. Fredrickson also carried out a characterization of antibiotic resistance in subsurface bacteria at the Hanford Site and the Savannah River Site. This research was performed at no cost to the funding agency because the student obtained an internal grant from Florida State University to support it. It is mentioned here only because it does add significantly to our knowledge of the characteristics of subsurface bacteria. Briefly, the bacteria from deep sediments at both sites were found to have surprisingly high frequencies of resistance and multiple drug resistance. These findings were published in *Microbial Ecology*. Additional studies indicated some of the Hanford isolated have a novel tetracycline resistance gene (Tet 42) that is very different from all previously describes

genes of this type. This work was published in *Antimicrobial Agents and Chemotherapy*. These findings raise interesting questions as why bacteria in the subsurface would have resistance to antibiotics to which they presumably have never been exposed.

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- Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka. 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). *Appl. Environ. Microbiol.* 69:7467-7479.

7. Products Delivered

7.1. Peer-Reviewed Journal Articles

1. Madden, A.S., A.C. Smith, D.L. Balkwill, L.A. Fagan, and T.J. Phelps. 2007. Microbial uranium immobilization independent of nitrate reduction. *Environ. Microbiol.* 9:2321-2330.
2. Fredrickson, J.K., S.M. Li, E.K. Gaidamakova, V.Y. Matrosova, M. Zhai, H.M. Suloloway, J.C. Scholten, M.G. Brown, D.L. Balkwill, and M.J. Daly. 2008. Protein oxidation: key to bacterial desiccation resistance? *ISME J.* 2:393-403.
3. Brown, M.G., and D.L. Balkwill. 2008. Antibiotic resistance in bacteria from the deep terrestrial subsurface. *Microb. Ecol.* (In Press).
4. Brown, M.G., E.H. Mitchell, and D.L. Balkwill. 2008. Tet 42, a novel tetracycline resistance determinant isolated from deep terrestrial subsurface bacteria. *Antimicrob. Agents Chemother.* 52:4518-4521.

7.2. Published Abstracts

1. Smith, A., T.J. Phelps, A. Fagan, and D.L. Balkwill. 2006. Microbial uranium immobilization independent of nitrate reduction. *Abstr. 106th Gen. Meeting Amer. Soc. for Microbiol.*, Orlando, FL.
2. Daly, M., S.M. Li, J. Scholten, E. Gaidamakova, V. Matrosova, D.L. Balkwill, J.K. Fredrickson, M.G. Brown, and H. Sulloway. 2007. Isolation and characterization of ionizing radiation-resistant soil bacteria from the Hanford Site, Washington. *Abstr. 107th Gen. Meeting Amer. Soc. for Microbiol.*, Toronto, Canada.
3. Brown, M.G., and D.L. Balkwill. 2007. Antibiotic resistance in bacteria from deep subsurface environments. *Abstr. 47th Interscience Conf. on Antimicrobial Agents & Chemotherapy*, Chicago, IL.
4. Brown, M.G., E.H. Mitchell, and D.L. Balkwill. 2008. A novel tetracycline resistance determinant, Tet 42, isolated from deep terrestrial subsurface bacteria. *Abstr. 108th Gen. Meeting Amer. Soc. for Microbiol.*, Boston, MA.