EMSP PROJECT NO. 86807

Long-Term Stewardship of Mixed Wastes: Passive Reactive Barriers for Simultaneous In Situ Remediation of Chlorinated Solvent, Heavy Metal, and Radionuclide Contaminants

This project report addresses the results of a 3-way collaboration between researchers at Montana State University's (MSU's) Center for Biofilm Engineering (CBE) (Drs. Robin Gerlach and Al Cunningham), the WSU/NSF IGERT Center for Multiphase Environmental Research (CMER) at Washington State University (WSU) (Dr. Brent Peyton who recently moved to MSU), and the Idaho National Laboratory (INL) (Drs. William Apel and Frank Roberto).

Each part of this project is funded under a different contract with the Office of Science of the US Department of Energy. Drs. Gerlach and Peyton recently requested one year no-cost extensions for their parts of the project since the initially anticipated start date for this project was delayed and hiring of qualified personnel was delayed in turn.

Background -Subsurface environments contaminated with radionuclides pose difficult remediation challenges. According to the National Research Council (*Research needs in Surface Science,* 2000. Nat. Acad. Press, Washington D.C.), cleanup across the DOE complex is expected to cost at least $200 billion dollars and will take decades to complete. Several methods are currently being used to treat U-contaminated groundwater, but all are expensive. An alternative to these technologies is the use of indigenous subsurface bacteria for immobilizing U in contaminated groundwater and soil. Three basic mechanisms by which bacteria can immobilize U are the following: 1) direct and indirect microbial reduction of U(VI) to U(IV) 2) uptake and accumulation by cells and 3) precipitation of U(VI) as uranyl phosphate with inorganic phosphate released by cells.

Strain ES6 is a Gram positive isolate from subsurface cores obtained from the DOE Hanford site in Washington State. Viamajala et al.(in review) showed that a majority of isolates enriched from Hanford cores contaminated with Cr and U, and from uncontaminated overlying sediments, were Gram positive facultative anaerobes in, or closely related to, the genus *Cellulomonas*. Sani et al. (Appl Microbiol Biotechnol (2002) 60:192–199) reported that *Cellulomonas* spp. were capable of removing Cr(VI) and U(VI) from solution in both the presence and absence of electron donors, and Borch et al. (2005) showed that strain ES6 is also capable of reducing nitroaromatics and ferrihydrite. Compared to Gram-negative bacteria, only a few Gram-positive organisms have been examined for metal-reduction capabilities as possible contributors to *in situ* metal bio-immobilization remediation strategies. Thus, the study of metal transformations catalyzed by *Cellulomonas* is environmentally relevant, particularly to the DOE Hanford site, and provides needed information on metal biotransformations of Gram positive organisms. Results presented here show for the first time that a subsurface *Cellulomonas* sp. can precipitate U by multiple mechanisms, namely by the release of inorganic phosphate and by U(VI) reduction in the presence and absence of anthraquinone-2,6-disulfonate (AQDS).

Research Objectives

The collaborative project was designed to evaluate the possibility developing a subsurface remediation technology for mixed wastes at Department of Energy sites using a group of common soil bacteria of the genus Cellulomonas. We have been gaining a better understanding of microbial transformation of chromium, uranium, iron minerals, and trinitrotoluene (TNT) by *Cellulomonas* spp. in simulated subsurface environments.

The project team investigated:

1) the influence of electron donors and acceptors on the reductive transformation of mixed contaminants in order to determine the most efficient way to utilize the activity of this group

of facultative anaerobes capable of reductive transformation reactions under fermentative conditions.
2) the long term activity of *Cellulomonas* spp. in the absence of external carbon sources.
3) the characterization of uranium immobilization strategies used by *Cellulomonas* spp. that may be stimulated in subsurface environments to produce a reactive U barrier.
4) the presence of *Cellulomonas* spp. at a number of DOE sites.

Research Progress and Implications

As of September 2005, MSU's efforts have demonstrated that strain ES6 (most closely related to *Cellulomonas hominis*) can reduce Cr(VI) and TNT in the presence and absence of a supplemental carbon source, electron shuttling compounds, and iron minerals. A carbon source screening identified molasses as a very efficient stimulator for Cr(VI) and TNT reduction activity. The presence of electron shuttles such as humic substances or anthraquinone disulfonic acid as well as iron minerals can enhance reduction rates and influence transformation pathways significantly. While the competition of oxidized iron minerals for the electrons needed for contaminant reduction appears to be minimal, the presence of previously reduced iron minerals increases contaminant reduction rates drastically demonstrating a chemical reaction between surface-associated ferrous iron and the oxidized contaminants.

In order to more closely investigate the mechanisms of Cr(VI) reduction, sucrose was chosen as the carbon source for most of the investigations so that the metabolic pathways involved in carbon source oxidation and reductive contaminant transformation can be investigated in more detail. The Cr(VI) reduction studies with starved cultures of strain ES6 indicated that the number of viable cells and the Cr(VI) reduction activity is decreasing with increasing starvation time but that the Cr(VI) reduction activity can be restored by the addition of sucrose as a carbon source. The lag time until Cr(VI) reduction observed increases with increasing starvation time. The results so far indicate that strain ES6 has some potential for long lasting Cr(VI) reduction activity.

In 2003, Dr. Gerlach provided support for a Ph.D. student (Thomas Borch) who investigated the transformation of the explosive 2,4,6-Trinitrotoluene (TNT) by strain ES6 in the presence of electron shuttling compounds and iron minerals. Dr. Borch is now a postdoctoral researcher at Stanford University and will begin an appointment as Assistant Professor in Soil Sciences at Colorado State University in March 2006. Five undergraduate research assistants and one technician were also supported by the project.

Meso-scale (4 ft length) column studies investigating the combined effects of direct and indirect microbial Cr(VI)-reduction were performed in a collaborative effort between MSU, WSU, and the INL in 2003. The columns studies ran over a period of several months and the results of these experiments form the basis for a manuscript currently in preparation (see below).

The results of the studies summarized above are either accepted for publication or are being prepared for publication in peer review journals. We anticipate a total of eight peer reviewed publications with MSU participation as a result of this project by the end of the no-cost extension period (i.e. by September 2006). Nine poster and platform presentations have been given as a result of the research conducted by Dr. Gerlach's MSU team.

At WSU, removal of uranium (U) from aqueous solution was studied using *Cellulomonas* sp. strain ES6, under anaerobic, non-growth conditions. The *Cellulomonadaceae* are environmentally relevant subsurface bacteria, and strain ES6 was isolated from the DOE Hanford subsurface. To better understand the role of the pH buffer in the U immobilization process, both bicarbonate and PIPES buffers were used. Our results show for the first time the strain ES6 has multiple U immobilization mechanisms within one organism.

During aerobic growth, strain ES6 accumulates excess phosphate as polyphosphate, which can be hydrolyzed and released as inorganic phosphate (P_i) under anaerobic starvation conditions. Inorganic phosphate released by the cells precipitated U(VI) as uranyl phosphate. The saturation concentration of phosphate required to initiate U precipitation was dependent on the buffer and amount of U in solution.

This long-term release of P_i can serve as a slow release reactant for U immobilization, thus the creation of a *Cellulomonas* biobarrier may have advantages of lower maintenance costs than may be necessary using dissimilatory metal reducing bacteria.

A Monod-based kinetic model described P_i release rates. This quantification is important to understanding this process in the field since the kinetics can be used in simulation models to estimate P_i release rates and thus U immobilization rates associated with a biobarrier.

Examination of the cultures by high-resolution transmission electron microscopy (HR-TEM) and energy dispersive X-ray spectroscopy (EDS) at the Pacific Northwest National Laboratory (PNNL) Environmental Molecular Sciences Laboratory (EMSL) showed both extracellular and intracellular U accumulation. The uranyl phosphate precipitates were nanometer sized needle-like fibrils and EDS analysis suggested a 1:1 molar ratio of U and phosphorus in these precipitates. Studies of U with strain ES6 in the presence of anthraquinone-2,6-disulfonate (AQDS), a model humic substance, showed that with AQDS present, U reduction becomes a more important removal mechanism than precipitation by phosphate ligands. X-ray absorption near-edge spectroscopy (XANES) analysis showed the predominant oxidation state of U precipitates was +4 in bicarbonate buffer and in AQDS treatments, but was +6 in PIPES buffer. Uranium immobilization by *Cellulomonas* sp. was previously reported to be solely by reduction, however present work suggests that strain ES6 can precipitate U via both precipitation with phosphate ligands and enzymatic reduction, depending on environmental conditions.

The results obtained to date are the first report of an environmentally relevant subsurface microorganism capable of U immobilization by two different mechanisms (reductive precipitation or precipitation with phosphate ligands) depending on environmental conditions. The project demonstrated a potential role for Gram positive organisms, represented here by the genus *Cellulomonas*, for immobilizing U(VI) as uranyl phosphate as well as U(VI) reduction. The ability of *Cellulomonas* sp. to reduce Cr(VI) to Cr(III), and to precipitate U as U(IV) and uranyl phosphate indicates a potential long-term application of in situ biological barriers for mixed heavy metal and radionuclide removal.

At INL *Cellulomonas sp*. strain ES6 was tested for anaerobic growth and hexavalent chromium reduction the presence of carbon tetrachloride (CT) in simulated ground-water medium with lactate, glycerol, and sucrose as sole carbon sources and electron donors. All electron donors were tested at an initial concentration of 10 mM and CT and hexavalent chromium were present at initial concentrations of 0.5 mg/L. There were no added electron acceptors or electron shuttles.

Only sucrose supported growth and chromium reduction under these conditions. In 42 hours cell numbers had increased from approximately 5×10^6 to 8×10^8. In the same period, approximately 20% of the hexavalent chromium was reduced versus abiotic controls. No reduction in CT levels was noted versus abiotic controls.

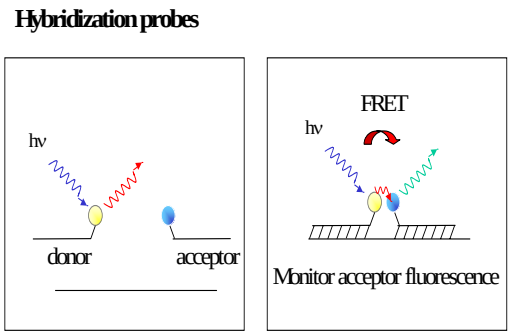
Real-time PCR (polymerase chain reactions) assays were designed to detect and quantify the presence of *Cellumonas* spp. in natural samples and laboratory studies where *Cellumonas* strain ES6 was used as inoculum. An assay specific to ES6 was designed as well as a universal *Cellumonas* assay to facilitate tracking of these populations. These assays were validated using a library of nearest neighbors, determined by 16S rRNA phylogeny (see Table 1). Assays were also validated against a broader panel of microorganisms including *E. coli* and a *Staphylococcus* sp. Hybridization probes were used as the method of fluorescence detection (Figure 1). Use of this detection approach allowed design of highly specific assays.

Table 1. Panel of DNAs used to validate real-time PCR assays.

Culture
ES-6
Oerskovia turbata
Oerskovia jenensis
Cellulomonas fermentans
Cellulomonas hominis
Dermabacter hominis

The optimized ES6-specific assay detected only ES6 and exhibited a detection limit of roughly 20 genomic copies. The universal *Cellumonas* assay exhibited minor cross-reactivity with *D. hominis*, but could potentially be further optimized to eliminate this interference.

Figure1. Real-time PCR detection approach.



Core ID	Well ID	Core Depth, meters
1B	C4131	37.0-37.5
2A	C4131	40.5-41.0
4A	C4131	44.5-45.0
5B	C4131	46.5-47.0
5C	C4131	46.0-46.5
7B	C4131	51.5-52.0
8A	C4131	54.5-55.0
1B*	C4132	37.5-38.0
2A	C4132	40.5-41.0
3B	C4132	42.0-42.5
4A	C4132	45.0-45.5
5B**	C4132	47.0-47.5
5C	C4132	45.5-47.0
6A	C4132	50.0-50.5
7B	C4132	52.0-52.5
8A	C4132	55.0-55.5

* "Carlos 1" core positive by microarray;
**"Carlos 5" positive by microarray

Table 2. Hanford Area 100 Core Samples Analyzed by Real-time PCR using ES6

Cellumonas spp. had previously been detected in DNA microarray studies of Hanford cores from the Ringold formation (Area 100) performed at LBNL. Sixteen core samples were received in our lab (see Table 2), including two that had positive microarray hits for *Cellulomonas*. The core material was aseptically crushed and processed to extract DNA. These DNA samples were subsequently analyzed using the ES6-specific real-time PCR assay to determine the presence or absence of this microorganism. None of the samples were positive for ES6. However, since the sequence target on the microarray was directed towards *Cellulomonas* strain K3 (closely related to *C. fermentans*), it is possible that the universal real-time PCR assay might be more appropriate for reanalysis of these core samples once it has been further refined to eliminate cross-reactivity with *D. hominis*.

Planned Activities

The one year no cost extension for Dr. Gerlach's and Dr. Peyton's parts of the project will be used to complete the six remaining manuscripts and write a separate final report for MSU's part of the project.

Information Access

Publications:

BORCH, T.; INSKEEP W.P.; HARWOOD, J.A.; R. GERLACH, R. (2005): Impact of Ferrihydrite and Anthraquinone-2,6-Disulphonate on the Reductive Transformation of 2,4,6-Trinitrotoluene by a Gram Positive Fermenting Bacterium. *Environmental Science and Technology*. 39, 18:7126-7133.

BORCH, T.; BIEDERMAN, J.A.; MOGK, D.W.; GERLACH, R.; BUTTERFIELD, P.W.; JORDAN, R.N.; CAMPER, A.K.: Characterization of Two Iron Oxide Analogs for Environmental Research: An Evaluation of Imaging and Analytical Techniques. Water Research. Submitted for publication.

VIAMAJALA, S., W. A. SMITH, R. K. SANI, B. M. PEYTON, W. A. APEL, J. N. PETERSEN, AND A. L. NEAL. Isolation and characterization of Cr(VI) reducing Cellulomonas spp. from subsurface soils: implications for long term chromate reduction. Bioresource Technology (In review)

BALLOR, N.R; GERLACH, R. Mixed Waste Degradation by Gram Positive Subsurface Bacteria and the Influence of Electron Shuttles. *Environmental Science and Technology.* In Preparation.

GERLACH, R.; VIAMAJALA, S; JENNINGS, L.K.; PEYTON, B.M.; APEL, W.A.; CUNNINGHAM, A.B. Influence of Carbon Sources and Electron Shuttles on Ferric Iron Reduction by *Cellulomonas* sp. Strain ES6. *Biodegradation*. In Preparation.

SIVASWAMY, V.; PEYTON, B.M.; VIAMAJALA, S.; GERLACH, R., APEL, W.A.; SANI, R.K.; DOHNALKOVA, A; BORCH, T. Multiple Mechanisms of Uranium Immobilization by Cellulomonas sp. strain. ES6. Environmental Science and Technology. In Preparation.

GERLACH, R.; FIELD, E.K.; PEYTON B.M.; APEL W.A.; CUNNINGHAM A.B. Influence of Carbon Source, Iron Minerals, and Electron Shuttling Compounds on the Bacterial Reduction of Chromate. In Preparation.

VIAMAJALA S., GERLACH R., SIVASWAMY V., PEYTON B.M., APEL W.A., CUNNINGHAM A.B., PETERSEN J.N. Chromate reduction by Cellulomonas: Multi-scale flow cell experiments. In Preparation.

BORCH T.; GERLACH R. & INSKEEP W.P. The Influence of Fe(Hydr)oxide Phases and the electron shuttle anthraquinone-2,6-disulfonate on the reduction of 2,4,6-Trinitrotoluene by a fermenting Bacterium. In Preparation.

Presentations:

FIELD, E.K., JENNINGS, L.K., CUNNINGHAM, A.B., PEYTON, B.M., GERLACH, R., AND APEL, W.A. (2005): Influence of Carbon Source, Iron Minerals, and Electron Shuttling Compounds on the Bacterial Reduction of Chromate. Poster Presentation at the INRA Environmental and Subsurface Science Symposium, Big Sky, MT, September 19-21, 2005

SIVASWAMY, V.; PEYTON, B. M. ; VIAMAJALA, S. ; GERLACH, R.; APEL, W.A.; SANI, R.; A. DOHNALKOVA, A. (2005): Multiple Mechanisms of Uranium Immobilization by Cellulomonas. Platform Presentation. Presentation at the INRA Environmental and Subsurface Science Symposium, Big Sky, MT, September 19-21, 2005

GERLACH, R.; BORCH, T.; BALLOR, N.R.; CUNNINGHAM, A.B.; PEYTON, B.M.; APEL, W.A. (2005): Fermenters and Reductive Contaminant Transformation Processes in the Subsurface. Platform Presentation. The Joint International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII). Jackson Hole, WY. August 14-19, 2005.

SIVASWAMY, V.; PEYTON, B. M. ; VIAMAJALA, S. ; GERLACH, R.; APEL, W.A.; SANI, R.; A. DOHNALKOVA, A. (2005): Uranium Immobilization by Cellulomonas. Platform Presentation. The Joint International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII). Jackson Hole, WY. August 14-19, 2005.

GERLACH, R.; BORCH,. T; CUNNINGHAM, A.B.; VIAMAJALA, S.; PEYTON, B.M.; APEL, W.A. (2004): Influence of Electron Shuttling Compounds and Iron Minerals on the Reduction of Chlorinated and Recalcitrant Compounds, Monterey, CA, May 24-27, 2004.

BALLOR, N.R.; GERLACH, R (2004): Bioremediation and the Role of Mixed Pollution: TNT and Cr(VI). Poster Presentation at the 2004 Annual Meeting, American Institute of Chemical Engineers. Austin Convention Center, Austin, TX. November 7-12, 2004

BALLOR, N.R.; GERLACH, R (2004): Bioremediation and the Role of Mixed Pollution: TNT and Cr(VI). Poster Presentation at the 2004 Fall Meeting of the Michigan American Society for Microbiology. Crystal Mountain Resort, MI, October 8/9, 2004

BORCH, T.; GERLACH, R.; CUNNINGHAM, A.B. (2003): Iron (III) Minerals Can Impact the Microbial Reduction of 2,4,6-Trinitrotoluene. Platform Presentation at the 3rd INRA Subsurface Science Symposium, Salt Lake City, UT, October 05-08, 2003

GERLACH, R.; BORCH, T.; CUNNINGHAM, A.B.; VIAMAJALA, S.; PEYTON, B.M.; APEL, W.A. (2003): Influence of Electron Shuttling Compounds on the Reduction of Metals and Organics. Platform Presentation at the 3rd INRA Subsurface Science Symposium, Salt Lake City, UT, October 05-08, 2003

VIAMAJALA, S.; GERLACH, R.; PEYTON, B.M.; APEL, W.A.; CUNNINGHAM, A.B.; PETERSEN, J.N. (2003): Passive Reactive Barrier (PRB) for In Situ Remediation of Cr(VI): Bench- and Meso-Scale Tests using Cellulomonas spp. Platform Presentation at the 3rd INRA Subsurface Science Symposium, Salt Lake City, UT, October 05-08, 2003