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Identification and Isolation of Active, Non-Cultured Bacteria from Radionuclide and Metal Contaminated Environments for Genome Analysis

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The **overall goal** of this project is to identify novel, previously uncultured groups of bacteria that may play important roles in bacterial community function in contaminated sediments and soils, and to obtain genomic DNA of members of these bacterial groups for genome analysis. Our studies have focused on non-cultured members of the *Acidobacteria* division. Members of this division are widespread in contaminated and pristine soils having vastly different physical and chemical characteristics, and they have been found to represent a major fraction of the non-cultured bacteria in several soils (by 16S rRNA clone library analysis). Their functions in soils and sediments are unknown.

The **four current objectives** of this project are to (1) identify active members of the *Acidobacteria* division that are present in radionuclide and metal contaminated subsurface sediments, (2) compare composition of *Acidobacteria* groups in soils as determined by DNA- or RNA-based methods, (3) attempt to culture novel *Acidobacteria* from soil environments, using a microcapsule approach, (4) obtain whole genome sequence from two *Acidobacteria* division species that belong to phylogenetic groups that are relatively abundant and active in sediment or soil environments.

(1) *Acidobacteria* groups in radionuclide contaminated subsurface environments. Comparisons of the relative abundance and composition of *Acidobacteria* in contaminated subsurface sediments from the NABIR FRC were conducted using PCR amplification of sediment DNA followed by cloning and sequencing. Two subsurface samples from the background area and at least 6 samples from 3 contaminated areas were compared. Both the contaminated and background sediments were dominated by members of the *Proteobacteria, Acidobacteria, Firmicutes, Actinomycetes* divisions, and unclassified species. The *Acidobacteria* comprised 15 to 26% of the total bacterial sequences in the backgrounds sites and 5 to 13% in the contaminated sites.

Within the *Acidobacteria* division, there are currently 8 described subgroups (groups 1 to 8). Through our subsurface sediment study, we have identified at least 7 new subgroups (groups 9 to 15). In the backgrounds sites, the most abundant groups were 1, 3, 4, 5, 6, 10. In the contaminated sites, new groups 9, 10, and 13 were very abundant, in addition to some of the original groups. This result demonstrates a dramatic shift in species composition within the *Acidobacteria* division from the

backgrounds to contaminated sites. Fine scale comparisons of phylotype diversity in the backgrounds and contaminated sites are in progress.

In addition to the FRC surveys, clone/sequence-based surveys of total bacteria and *Acidobacteria* division members were conducted on samples from the Rifle Site, CO and a deep subsurface site at PNNL. At the Rifle site, the *Proteobacteria, Acidobacteria, Firmicutes* and unclassified species were most abundant in clone/sequence libraries. As with most of the surface soils we have characterized, the Rifle site samples primarily contained *Acidobacteria* groups 4 and 6. PNNL site results are in analysis now.

(2) DNA- vs. RNA-based analyses for assessing bacterial community structure. A quantitative, real-time PCR assay for *Acidobacteria* was developed to determine the proportion of *Acidobacteria* relative to total bacteria in environmental samples. We found this assay to work well with surface soils, but had difficulty in subsurface samples from the NABIR FRC due to very low DNA concentrations. We also refined RNA extraction protocols and a reverse transcriptase (RT)-PCR method to assess the presence of 'active' *Acidobacteria* (by interrogation of the RNA) in soil and subsurface sediment samples. A comparative study of total bacteria and *Acidobacteria* division members was conducted in soil using DNA PCR to assess relative abundance of 16S rRNA gene sequences, and using RNA RT-PCR to determine active members (or those with the most ribosomes). Comparisons were made using a T-RFLP method designed for the *Acidobacteria*, and clone/sequence analysis. Sequences from *Acidobacteria* groups 1, 4 and 6 were found to be the most numerous by DNA analysis. However, in the soil tested, groups 1, 3, and 5 were most active by RNA analysis Follow up experiments are focusing on the relative activity and abundance of groups 3, 4, 5, and 6.

(3) Attempts to culture group 6 *Acidobacteria* using Diversa Corporation microcapsule technology. In collaboration with Martin Keller and Karsten Zengler at Diversa Corp., San Diego, CA, and Fred Brockman at PNNL, we are attempting to culture group 6 *Acidobacteria* using Diversa's microcapsule / dilute culture / flow sorting techniques. The first attempt to culture *Acidobacteria* was partially successful, in that we were able to detect them in microcapsules, but were not able to grow them to high titer. Our current attempts involve detection at the microcapsules stage, followed by rolling-circle, whole genome amplification (which can be accomplished from a few to a 100 cells), to generate genomic DNA.

(4) Whole genome sequencing of soil-borne *Acidobacteria*. In collaboration with Peter Janssen, Univ. of Melbourne, Australia, we are providing DNA to the JGI for whole genome sequencing of two *Acidobacteria*, one from group 3 and one from group 4 or 5. These cultures are extremely slow growing and DNA yields are less than 5 μg. The JGI is currently generating libraries from the group 3 isolate.

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Metagenomic Analysis of Uncultured *Cytophaga* and Other Microbes in Marine and Freshwater Consortia

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Most bacteria and archaea in natural environments still cannot be isolated and cultivated as pure cultures in the laboratory, and the microbes that can be cultured appear to be quite different from uncultured ones. Consequently, the phylogenetic composition, physiological capacity and genetic properties of natural microbes have to be deduced from bulk properties of microbial assemblages, fluorescence in situ hybridization (FISH) assays, and from a variety of PCR-based methods applied to DNA isolated directly from natural samples. Another culture-independent approach is to clone this DNA directly into appropriate vectors and to screen the resulting "metagenomic library", which theoretically consists of all possible genes from the microbial assemblage. We applied this general approach to the freshwater end of the Delaware Estuary and to the western Arctic Ocean as part of our efforts to understand carbon and nitrogen cycling in environments like estuaries with large environmental gradients. Metagenomic libraries have been constructed for soils and some marine samples, but not for freshwaters nor for a high latitude ocean. High molecular weight DNA from the bacterial size fraction was isolated and cloned into the fosmid vector pCC1FOS (Epicentre). Our libraries consisted of about 5000 clones with an average insert size of 40 kB, representing about 90 genomes, if we assume a genome size of 2 mB.

Screening the libraries revealed several surprises, including genes found previously in metagenomic libraries of oceanic samples. The Delaware River library appears to be dominated by *Cytophaga*-like bacteria according to the 16S rRNA data collected by DGGE analysis of PCR amplified 16S rRNA genes. Of the 80 clones bearing 16S rRNA genes, about 50% appear to be from the *Cytophaga-Flavobacteria*, a complex cluster in the Bacteroidetes division. The complete sequence of a fosmid clone containing a *Cytophaga*-like 16S rRNA gene will be presented. FISH analysis of the original microbial assemblage indicated that *Cytophaga*-like bacteria were only about 15% of the community. The next most abundant 16S rRNA genes in the library are from G+ *Actinobacteria*, which others have shown to be abundant in freshwater lakes. But beta-proteobacteria usually dominate freshwater systems and were the most abundant group in our sample according to the FISH analysis, yet beta-proteobacteria accounted for only about 15% of the 16S rRNA genes in the metagenomic library, much less than the 25% found by FISH. Estimates of species-level diversity obtained by rarefaction analysis of fosmid clones bearing 16S rRNA genes differed substantially from clones of PCR products with and without suppression of heteroduplex formation. 16S rRNA gene diversity in the metagenomic library indicated that species-level diversity in this freshwater environment may be on the order of tens of species, much less than current estimates.

We also screened the library for genes indicative of a newly-discovered photoheterotrophic metabolism, aerobic anoxygenic photosynthesis (AAnPS). Marine bacteria carrying out AAnPS contain photosynthesis genes that cluster with those from alpha-, beta-, and gamma- proteobacteria. To date, the diversity and expression of uncultured AAnPS genes in temperate freshwaters have not been examined. We surveyed the Delaware River for *pufL* and *pufM* genes, which encode

AAnPS reaction center proteins, in the fosmid library. Two fosmid clones containing AAnPS photosynthetic operons were completely sequenced and annotated. The operons in the two clones were organized differently than known cultured and uncultured organisms from marine and freshwaters. One clone contained genes most closely related to those of beta-proteobacteria. Preliminary data on *pufM* genes amplified from DNA of Delaware estuary bacteria suggest that most of those genes were most closely related to those of beta-proteobacteria. PCR-amplified and genomic-isolated *pufM* genes cluster separately from currently known cultured and uncultured AAnPS. These data on *pufM* genes in the Delaware estuary indicate an unexpected diversity of estuarine AAnPS bacteria and can be used to explore their ecological success during the transit through the estuary into coastal waters.

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Approaches for Obtaining Genomic Information from Contaminated Sediments Beneath a Leaking High-Level Radioactive Waste Tank

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The SX Tank Farm at the US Department of Energy's Hanford Site in Washington state was built in 1953 to receive high level radioactive waste, and consists of dozens of one million gallon enclosed tanks. The waste resulted from recovery of purified plutonium and uranium from irradiated production fuels using methyl isobutyl ketone, aluminum nitrate, nitric acid, and sodium dichromate. Between 1962 and 1969, tens of thousands of gallons of radioactive liquid leaked from tank SX-108. An extreme environment formed in the vadose zone from incursion of radioactive, caustic, and toxic contaminants and heating from the self-boiling contents of the tank. Samples from beneath the leaking tank were heated in some cases to above 100 degrees Centigrade, contained up to 50 microCuries of Cesium-137 per gram sediment, lower concentrations of other radionuclides, nitrate at 1% to 5% of sediment mass, and pH's to 9.8. These samples are the most radioactive sediments studied to date at the DOE Hanford Site. We hypothesized these extreme conditions would result in a relatively non-diverse community containing novel uncultured microbial divisions.

The original goal was to create a fosmid/BAC library and perform sequencing of clones representing novel uncultured microbial divisions, or less targeted sequencing to characterize the community as a whole. Low biomass levels (10 ^ 5 cells/gram and lower) in combination with very high radioactivity precluded the purification of DNA from the 10's of kilograms of sediment that was required to obtain microgram quantities of DNA for fosmid/BAC library construction. Moreover, extensive characterization of amplified and cloned 16S sequences, from 8 sediments and 30 enrichments from the sediments, failed to show the presence of novel uncultured microbial divisions. Never the less, the 16S rDNA sequencing identified over 40 different genera in the sediments. Approximately 75% of these genera were from the high G+C Gram positive division, highlighting the ability to survive simultaneous extreme conditions for 30-40 years is a widespread trait in this phylogenetic group.

Because the similarity scores of the 16S rDNA clones were mostly >0.95 with known cultured organisms, we elected not to pool enrichments and construct and sequence a fosmid/BAC library. Instead, the project was refocused on (1) an alternative technology for characterizing microbial communities and (2) shotgun sequencing of genomic DNA derived from pooled enrichments.

The first approach involves a novel high throughput microcapsule cultivation method that has been shown to allow culturing of some previously uncultivated microorganisms, allows 16S rDNA characterization, and can be coupled to whole genome (rolling circle) amplification for genome sequencing from the microcolony-containing microcapsules (see poster at this meeting by Zengler et al.). Key aspects of this technology are that it enables propagation of single organisms with extremely slow growth rates and low maximum cell densities, and preserves some of the community interactions and other specific requirements needed for successful cultivation. This approach allows direct access to physiological and genomic information from uncultured and/or difficult-to-culture microorganisms, and is thus fundamentally different than indirect access via shotgun or BAC clones derived from community nucleic acids. Specifically, the units of analysis are living, pure (or nearly pure) microcolonies, as opposed to the disassembled mixture of small fragments of genomes that have lost their biological context in studies using community nucleic acids.

For characterization by the microcapsule culturing approach, the 16 cores were stratified into 4 environmental zones based on levels of radioactivity, temperature, nitrate, and chromium. For each environmental zone and for a nearby uncontaminated borehole, sediments were pooled and cells purified with multiple nycodenz cushion centrifugations. The final cell preparations (from 25 g sediment) contained a total of $3x10^4$ to $9x10^5$ cells by AO counting. The 5 cell preps were each encapsulated to isolate individual cells into microcapsules, the community reconstituted by placing gmd's into a column, and diluted soil extract pumped through the columns to promote slow growth. Three culture conditions were used for each of the 5 cell preps: a high concentration of a mixture of soil extracts under oxic conditions, a high concentration of the same under microaerophilic conditions, and a low concentration of the same under oxic conditions. After several weeks of growth, microcapsules were analyzed by flow cytometry to identify those containing a microcolony, and positives were individually sorted into microtiter wells. A subsample of 2,900 putative microcolony- containing microcapsules from each cell prep were randomly selected for further analysis. The 14,500 cultures were grown further and screened by high throughput FT-IR spectroscopy. Cluster analysis was performed on spectra to identify clusters. Seven to 35 clusters were found per culturing condition per cell preparation from beneath the tank, and 18-90 clusters were found per culturing condition per cell preparation from the uncontaminated sediments. The 16S rRNA gene was sequenced for a representative of each cluster and phylogenetic analysis is ongoing.

The genera obtained by microcapsule culturing will be compared to the approximately 50 isolates previously obtained by plate and liquid culturing. One or more of the most unique microbes (<0.85 similarity to 16S sequences in databases) will be characterized by partial genome sequencing.

For the second approach, the DOE Production Genomics Facility has performed first-pass shotgun sequencing of genomic DNA from enrichments from sediments most highly impacted by the tank waste. Protein hit results will be presented at the meeting.

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Application of High Throughput Microcapsules Culturing to Develop a Novel Genomics Technology Platform

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Project Description

The overall goal of this proposal is to demonstrate the combination of high-throughput cultivation in microcapsules, which gives access to previously uncultivated microorganisms with genome sequencing from one to a few microcolony-containing microcapsules. This will allow direct access to physiological and genomic information from uncultured and/or difficult-to-culture microorganisms. This approach is fundamentally different than characterization and/or assembly of shotgun or BAC clones derived from community DNA or RNA. The units of analysis in our approach are living, pure microbial cultures in microcapsules, as opposed to the disassembled mixture of small fragments of genomes and cellular networks that have lost their biological context in studies using community nucleic acids. It is envisioned that the microcapsule based, high-throughput cultivation method will also be combined with Proteomics technology in the future.

Overall Goal

The overall goal is to prove that microcolonies of previously uncultured microbes derived through this high-throughput cultivation method are sufficient to create genomic sequence information.

The specific goals of this proposed work are:

1. Apply a high-throughput, microcapsule based cultivation technology to capture novel, previously uncultured microbes from a prairie soil relevant to DOE's mission in carbon sequestration.

2. Optimize fluorescent in situ hybridization (FISH) methods to selectively target and sort encapsulated microcolonies of interest using high speed fluorescence activated cell sorting.

3. Employ whole-genome amplification techniques to acquire a sufficient mass of DNA from targeted, encapsulated microcolonies to generate libraries for shotgun sequencing of entire genomes.

4. Develop sensitive methods to amplify specific mRNAs from targeted microcolonies that have been exposed to varying, environmentally relevant conditions.

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Insights into Community Structure and Metabolism Obtained by Reconstruction of Microbial Genomes from the Environment

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Microbial communities play vital roles in the functioning of all ecosystems. However, the vast majority of microorganisms are uncultivated, thus their roles in natural systems are poorly understood. Random shotgun sequencing of DNA from entire microbial communities is one approach for recovery of the gene complement of uncultivated organisms and for determining the degree of variability within populations at the genome level. Here we report reconstruction of near complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partial recovery of three other genomes from a natural acidophilic biofilm. This was possible with a modest sequencing effort because the biofilm was dominated by a small number of species populations and the frequency of genomic rearrangements and gene insertions or deletions was relatively low. Because each sequence read came from a different individual, we could determine that single nucleotide polymorphisms are the predominant form of heterogeneity at the strain level. The *Leptospirillum* group II genome had remarkably few nucleotide polymorphisms, despite the existence of a larger pool of low abundance variants. In contrast, we infer that the *Ferroplasma* type II genome is a composite of three ancestral strains that have undergone homologous recombination to form a population of many thousands of unique mosaic genomes. Analysis of the gene complement for each organism revealed the pathways for carbon and nitrogen fixation and energy generation and provided insights into survival strategies in an extreme environment.

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Growing Unculturable Microorganisms from Soil Communities

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The aim of this project (starting Feb 1, 2004) is to develop methods for growing "unculturable" soil microorganisms. Microorganisms play an important role in shaping the biosphere by affecting biogeochemical cycles and global climate. Novel technologies based on the understanding of microbial life are likely to emerge in the fields of energy production and environmental cleanup, which are of particular interest to DOE. Further development of advanced technologies such as genomics, proteomics, systems biology will be critical to the success of the GTL program. Application of these methods will require access to microorganisms, of which the vast majority, ≥99%, remain "unculturable". Rapid advancement in the understand-

ing of microorganisms and their communities will therefore depend critically on our ability to grow them. The importance of gaining access to uncultivables is underscored by the fact that some of the DOE target sites *have not yielded a single cultivable microorganism*, even though microbial diversity of those environments is substantial. In many locations of the DOE Field Research Center that do produce culturable organisms, these appear not to be the dominant species involved in bioremediation.

The proposed research is based on a method we recently developed that allows for growth of unculturable bacteria by placing them in a simulated natural environment in a diffusion chamber (Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**: 1127-1129). We will use the sites at the DOE Field Research Center as a model to develop methods for growing microorganisms and studying microbial communities. This project will benefit from a considerable amount of information on microbial communities already obtained for these sites.

The **Goals** of this study are:

1. **Characterization of groundwater and soil microbial communities**. An examination of microbial communities will be performed, using molecular approaches. Knowledge of the species composition of these communities will be used to construct specific fluorescent probes. Applying these probes back to the original soil/groundwater samples will allow us to determine the numerically dominant species.

2. **Culturing microorganisms from microbial communities**. Samples of groundwater and soil will be taken from the ORNL sites, and used to culture organisms in a simulated in situ environment in diffusion chambers. Colonies in the chambers will be screened by ARDRA and individual species identified by 16S rRNA approach to verify the presence and growth of species dominating natural communities. We will also isolate organisms from ORNL environments that so far have rendered no cultivable species at all.

3. **In vitro growth of uncultured organisms**. In order to facilitate subsequent analysis, methods to grow uncultured species in vitro will be developed. We find that many isolates will grow on a Petry dish in the presence of another organism from the same environment. We will thus assemble a panel of suitable "helper" organisms that support growth of soil isolates on artificial media. This co-culture approach will serve as a starting point for eventual reconstruction in vitro of a functional soil microbial community, which will be done in our future studies. We have also noted that a majority of uncultured isolates can be adapted to growth in vitro after a number of passages through diffusion chambers. We will use this "domestication" procedure to soil isolates obtained in this study as well.

4. The interesting organisms will be isolated in pure culture and submitted for whole genome sequencing.