PROGRAM ELEMENT 3

Biomolecular Sciences and Engineering

Molecular and Microcosm Analyses of the Potential for Gene Transfer in Radionuclei and Metal-Contaminated Subsurface Environments

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The objectives of this project are to (1) determine if metal resistance among subsurface microbes evolved by gene transfer; (2) measure the potential for gene transfer in subsurface microbial communities; (3) use microcosms to measure transfer rate; and (4) determine if gene transfer increases the ability of the community to respond to metal and radionuclei stress.

These objectives are accomplished by testing the following hypotheses (in corresponding order to the listed objectives):

Among subsurface microbes the phylogenetic relationships of metal resistance genes do not correspond with the rRNA-based phylogeny of their host strains, indicating that metal resistance evolved by horizontal rather then vertical transfer.

Conjugal plasmids are more abundant in microbial communities of metal and radionuclei contaminated soils than in pristine soils.

The presence of metals and radionuclei stimulates conjugal transfer in the subsurface environment.

The spread of metal resistance via conjugal transfer increases the resilience of the subsurface microbial community to metal stress.

Hypothesis (1) is tested by comparing the phylogenetic relationships among subsurface culture collection strains and among metal resistance genes that are carried by these strains. The hypothesis will be accepted if the phylogenetic trees that emerge are dissimilar; similar trees will indicate a coevolution of microbes and their metal tolerance. Hypothesis (2) is tested by using the exogenous plasmid isolation approach. Genetically marked recipient strains are mixed with subsurface soils, isolated bacteria and DNA obtained from these soils, and metal resistant exconjugants are selected. The hypothesis will be accepted if conjugal metal resistance plasmids are more frequently isolated from contaminated as compared to pristine soils. Hypothesis (4) is tested by measuring conjugal transfer rates in metal-spiked subsurface soil microcosms. The hypothesis will be accepted if the transfer rate is stimulated in the presence of heavy metals. Hypothesis (4) is tested by evaluating genotypic, phenotypic and functional diversities of subsurface communities in the presence of metals. Microcosms containing a bacterium with a metal resistance mobilizable plasmid and a conjugative mobilizing plasmid are compared with microcosms containing the same bacterium with only the mobilizable metal resistance plasmid. If a community where transfer of the metal resistance genes occurs at high frequency shows higher diversity compared to a community where transfer is less frequent, the hypothesis will be accepted.

Metabolic Engineering of Microorganisms for Heavy Metal Bioremediation

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An attractive method for heavy metal remediation is precipitation of the metal on the bacterial cell wall as an insoluble metal complex. Two potential applications for this technology are immobilization of heavy metals in situ and improved flocculation and removal of metals from waste streams. While bioprecipitation of metals would not remove the metal from its environment, it could be used to slow migration of the metal into groundwater. In a reactor, bioprecipitation can take advantage of the improved settling ability of cells over non-biological metal complexes, and thereby decrease the cost of treating heavy metal-contaminated waste streams.

To this end, we have engineered the metabolism of Gram-negative bacteria (namely, *Escherichia coli* and *Pseudomonas aeruginosa*) to secrete sulfides or phosphates for precipitation of cadmium sulfide or uranyl phosphate, respectively, on the cell wall. Two aerobic pathways for sulfide secretion were developed. The first relies on reduction of thiosulfate to form sulfide. The second relies on deregulation of the cysteine biosynthetic pathway and subsequent production of sulfide from cysteine. For phosphate secretion, we engineered the polyphosphate pathways to improve polyphosphate accumulation under phosphate-rich conditions and its subsequent degradation to and secretion of orthophosphate under phosphate-starvation conditions. When secretion of phosphate or sulfide occurs, complete removal of the metal is observed, even at relatively high concentrations. Electron microscopy and energy dispersive X-ray spectroscopy confirmed the presence of the metal precipitate on the outside of the cell. Future work will involve its application to other metals that readily precipitate as phosphates or sulfides.

Engineering Deinococcus Radiodurans for Remediation of Radioactive Mixed Waste Environments

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Immense volumes of radioactive waste, generated from nuclear weapons production during the Cold War, were disposed directly to the ground. The current expense of remediating these polluted sites is driving the development of alternative remediation strategies using microorganisms. Deinococcus radiodurans is the most radiation resistant organism known and can grow in highly irradiating environments. D. radiodurans strains expressing cloned mercury(II) resistance functions were constructed and shown to be effective at reducing toxic ionic mercury, a frequent constituent of radioactive wastes, to volatile elemental mercury. The mer operon was chosen as a model-system to guide future engineering efforts, particularly in the area of metal remediation.

To demonstrate how future engineering efforts of mixed radioactive wastes could be achieved, mercury-reducing and toluene-metabolizing functions were combined into the same host, yielding a strain expressing both functions. We have analyzed D. radiodurans growth on a chemically defined minimal medium in the presence and absence of continuous radiation. Whereas cell growth was unaffected in the absence of radiation, cells did not grow, and were killed, under continuous radiation. In nutrient-limiting conditions, DNA repair was found to be limited by this organism's metabolic capabilities and not by any nutritionally induced defect in genetic repair. Our growth studies and analysis of the complete D. radiodurans genomic sequence support the existence of several defects in D. radiodurans' global metabolic regulation that limit carbon, nitrogen and DNA metabolism. We have identified key nutritional constituents that restore growth of D. radiodurans in nutritionally limiting radioactive environments.

Stabilization of Radionuclides by Anaerobic Bacteria

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The genus Clostridium is a diverse group of anaerobic, gram-positive, rod-shaped, endo-spore forming bacteria. The taxon is very heterogeneous, comprising organisms with considerable variation in genome size (2.5 to 6.5 Mb) and G+C content (24 to 55 mol%). *Clostridium* sp. BC1 (ATCC No. 53464; hereafter referred to as BC1, an anaerobic, N_2-fixing member of this group that was isolated from coal-cleaning residues, is a potential candidate for ameliorating radionuclide contamination at DOE sites as it has biochemical pathways that can convert water soluble uranyl ion U(VI) to less soluble U(IV). The objective of this project is to use a whole genome shotgun sequencing approach to obtain large amounts of BC1 DNA sequence information to discover key functional genes and to understand gene sequence/function relationships in this and related Clostridia/Bacillus species. We are currently sequencing several BC1 clone libraries constructed in a vector, pZIP, we developed for generating sets of bidirectional nested deletions. Fragments in the 10 kbp size range or larger can be sequenced on both strands from ordered sets of nested deletions using universal vector primers to produce highly accurate sequence contigs. We are in the process of annotating several of these contigs encoding genes in involved in intermediary metabolism and reductive reactions using a graphics-based gene finder program we developed to aid in analysis of sequence assemblies.

We are also sequencing DNA from *Alcaligenes eutrophus* (*Ralstonia eutropha*) CH34, a gram-negative, non-spore forming bacillus that flourishes in millimolar concentrations of heavy metals. Resistance is conferred by large megaplasmids carrying gene clusters that encode cation-efflux complexes which span both bacterial membranes. The reference stock CH34 harbors two plasmids, pMOL28 (180 kb) and pMOL30 (240 kb), which together confer resistance to Zn, Cd, Co, Pb, Cu, Hg, Ni and Cr. Both plasmids are low copy number, stably maintained even without selective pressure and are self-transferable at low frequencies. About 20% of each plasmid is used to encode metal resistance. Due to the activity of these efflux systems, a supersaturated zone of metals is formed around the cells, which leads to bioprecipitation or biomineralization of heavy metals on the cell envelopes and removal of heavy metals from solution. Other large plasmids in other *A. eutrophus* strains confer resistance to xenobiotics. *A. eutrophus* can use a broad range of substrates as its carbon source or it can grow chemolitho-trophically using molecular hydrogen as the energy source and carbon dioxide as a carbon source. In the presence of nitrate *A. eutrophus* can grow anaerobically. This combination of properties makes this bacterium another attractive candidate for application in several bioremediation scenarios relevant to DOE waste sites.

We are about halfway through sequencing of pMOL28. A remarkable feature of *A. eutrophus* CH34, whose optimal growth temperature is around 30°C, is a high rate of cell death and mutation that occurs when it is grown at 37°C. As part of this project we sequenced a 21.5 kb segment of the *Alcaligenes* chromosome that complements a mutant phenotype of temperature resistant growth at 37°C back to the wild-type temperature sensitive phenotype. In collaboration with D. van der Lelie from the Flemish Institute of Technological Research in Belgium, we are now carrying out experiments to define which of the 17 ORFs within this region causes temperature sensitive growth and the nature of the mutations that occur at elevated growth temperatures.

Characterization of Environmental Regulation of the Genes and Proteins Involved in Metal Reduction Pathways in *Shewanella Putrefaciens*

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Shewanella putrefaciens is a versatile microbe capable of metal reduction in both aerobic and anaerobic environments and is thus of interest for bioremediation of waste sites containing toxic metal compounds. Assessing the usefulness of *S. putrefaciens* for bioremediation, however, requires characterization of the molecular mechanisms and regulation of the metal reduction activity. The U.S. Department of Energy Microbial Genome Program is funding the sequencing of the *S. putrefaciens* genome and the development of microarrays containing all ORFs from *S. putrefaciens* MR-1. NABIR is now funding a project to correlate the output of these Microbial Genome projects with changes in protein expression as a means to characterize the regulatory mechanisms controlling metal reduction activity when *S. putrefaciens* is grown in different environmental conditions. Shifts in the abundance of specific proteins are indicative of gene regulation, while the relative abundance of chemically modified forms of proteins (i.e., phosphorylated, glycosylated, deamidated, or methylated) reveals mechanisms of metabolic pathway regulation.

In this new NABIR project, *S. putrefaciens* MR-1 cells are grown under experimental conditions designed to replicate metal contamination in a variety of pH and temperature environments. Messenger RNA and proteins are extracted from the cells and analyzed using microarrays and two-dimensional gel electrophoresis, respectively. Proteins altered in abundance in cells grown in the presence of metals will be identified and the correspondence between protein changes and changes in the expression of specific genes detected through the microarray analysis will be examined. Post-translational modifications will be characterized and the mechanism of regulation of protein function deduced. Based on identified changes in gene expression in different environmental conditions, knockout mutants will be generated to determine whether the regulated genes are essential for cell survival. The results of the proposed experiments will identify (1) gene sequences required for *S. putrefaciens'* metal reduction activity in a variety of environments, (2) regulatory pathways controlling the abundance of the gene products, and (3) the effects of those genes on cell viability.

Complete Genome Sequencing of *Shewanella Putrefaciens*

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Shewanella putrefaciens grows both aerobically and anaerobically. In the anaerobic phase, it acts as a metal reducer. The use of metal reducing bacteria in bioremediation has several advantages over more standard respiring bacteria, including: (1) their substrates (iron oxides) are solids and thereby can be delivered to a contaminated site without diffusing away; (2)iron oxides are specific substrates, so competition from other bacteria for the electron acceptor will be minimal; (3) in stratified aqueous environments, reduced iron should diffuse upward, be reoxidized by molecular oxygen in the oxic zone, and return to the anoxic zone by gravity, thus acting as a "pump" for oxidizing equivalents.

The 5 Mb *S. putrefaciens* MR-1 genome is being sequenced by the random whole genome strategy used to complete the sequence of multiple bacterial genomes at The Institute for Genomic Research. The random sequencing phase of the project resulted in 70,000 sequences. These were assembled into 125 linked contigs. Currently, gaps are being closed using a combination of PCR and small and large insert clone walking.

In-Situ Survival Mechanisms of Sulfate-Reducing Bacteria in Polluted Sediments

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The long-term goal of our work is to identify genes and physiological activities essential for the in-situ survival of sulfate-reducing bacteria. Specifically, we are delineating genes from *Desulfovibrio* strains that are essential to its survival within contaminated environments. Our approach allows direct profiling between genes essential for survival in different settings such as normal and contaminated environments. With this method we can identify essential genes which could be missed in standard laboratory media, and identify genes which may important for survival in the presence of specific contaminants (U, Co, As, Pb).

To carry out these studies we are exploiting approaches developed in microbial pathogenesis to characterize genes essential for survival in experimental mouse models, the pathogen in-situ environment. As a complementary approach, we are using an environmentally important microbe and contaminated sediments for our experimental model. This approach involves using a pool of Tn5 based transposons which have been designed to express unique random tags. Thus, every transposon has its own signature and the technique is termed signature-tagged mutagenesis (STM). With STM, transposons can be transformed into the target organism to generate a library of transposon insertions carrying unique tags. Since each mutant carries a unique tag, it can be followed during the selection process. An input pool of STM clones is generated under normal conditions and then grown under the selective conditions. Those that grow in the normal setting, but are lost in the contaminated setting must have transposon disruption in gene(s) important for dealing with that contaminant. This clone is then selected from the input pool and the insertional mutation is characterized.

We have recently started work on this project and are developing transformation protocols for *Desulfovibrio*. We have recently isolated a new *Desulfovibrio* strain from a uranium-contaminated subsurface location. This strain will be used as the model in these studies. Using a combination of approaches, including electroporation and conjugation, we are optimizing the conditions for generating competent cells and obtaining random transposon insertions.

Genes and Functions Regulated by adnA in *Pseudomonas fluorescens*

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The genetic traits that contribute to the survival and competitive fitness of bacteria in soil are poorly understood. One potentially important factor is the ability to adhere to abiotic surfaces, which are nutritionally rich relative to the interstitial spaces. The common soil bacterium *Pseudomonas fluorescens* adheres to sand columns. Screening of 3500 mutants generated by Tn*5*-insertion mutagenesis identified three strains that adhered < 50% as well as the wild type strain. The Tn*5* insertions in these three strains mapped to two genes, adnA and adnB; adnA is a transcription factor of the NtrC/NifA family based on sequence homology. Intriguingly it is most similar to fleQ of *P. aeruginosa* (82% identity), a transcription factor required for adhesion to epithelial cells and mucins. Strains with insertions in adnA lack flagella, suggesting that adnA regulates flagellar gene expression and perhaps other cell surface structures required for adhesion. Recent work indicates that AdnA expression alone can complement the defects in adhesion and motility. The strain with an insertion in adnB is hyperflagellated and has reduced mobility. adnB is similar to the flagellar motor protein motA, an integral membrane ion channel.

Our goals are to further examine the role of the AdnA by identifying genes that are activated or repressed by adnA, and to identify environmental conditions that regulate adhesion. To address the first goal, we are preparing a *P. fluorescens* strain in which wild type adnA will be replaced by a copy whose transcription requires an exogenous inducer. Briefly, a plasmid DNA was constructed in which adnA is transcribed from the promoter of the *meta*-cleavage pathway operon (Pm) of the *Pseudomonas putida* TOL plasmid pWWO. Transcription from Pm requires the activator protein XylS, and XylS activates transcription only in the presence of an added inducer, in this case 3-methylbenzoate (3-MB). The chromosomal adnA promoter will be replaced by the inducible promoter cassette in an otherwise wild type background. The new strain will be tested to insure that adhesion and motility are 3-MB dependent. The strain will then be mutagenized with Tn*5*-*lacZ* or Tn*5*-*phoA* transposons and screened for differences in reporter enzyme activity in the presence or absence of 3-MB. Inducer-dependent changes in activity will indicate that the insertion has occurred in a transcription unit whose expression is affected by AdnA. The disrupted genes identified will be candidates for genes that mediate sand adhesion and other traits needed for survival in soil.

Work is also proceeding on characterizing environmental conditions that regulate adhesion. Using a simplified adhesion assay, we find that wild type cells grown in rich or minimal media adhere to borosilicate glass. Various combinations of carbon sources and metal ions were added to minimal media. Preliminary results suggest that cells grown on glucose, glutamate or acetate media are adherent, while cells grown on citrate medium are nonadherent. Citrate is dominant if two carbon sources are used together. Additionally, calcium in the media promotes adhesion, while iron inhibits adhesion. These results provide initial data relating to the nature of the extracellular signals that regulate adhesion. An understanding of the mechanisms promoting or inhibiting adhesion will allow greater flexibility in designing genetically modified organisms suited to particular purposes.

Mechanisms for Uranium and Technetium Reduction in *Geobacter sulfurreducens*

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Project work was initiated on Sept. 1, 1999, and the following objectives were achieved in the first month. Mass culture of *Geobacter sulfurreducens* was optimized, allowing generation of sufficient quantities of biomass for initial characterization of the enzyme systems catalyzing U(VI) and Tc(VII). Assays suitable for monitoring the reduction of Tc(VII) and U(VI) were also developed. Finally, mg quantities of the 9.6 kDa c-type cytochrome were purified from the soluble fraction of *G.sulfurreducens*, and shown to reduce U(VI) in vitro.

Flow Cytometry Technique for Multiplexed Detection, Quantification and Isolation of Nucleic Acids

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The goal of this work is to develop a new polystyrene bead-based capture method to identify specific DNA sequences from mixtures of heterogeneous DNA samples obtained from environmental samples. The methodology uses beads impregnated with different colored fluorescent dyes. The beads are usually coupled to DNA oligonucleotides that are used as capture probes. Following capture of complementary DNA sequences from the environmental samples, the fluorescent beads can be analyzed and separated by flow cytometry. The bead-based method may provide information comparable to information obtained using DNA microarrays, although with potential advantages such as greater sensitivity, quantitation and sequence determination. The method can also be preparative; primers based on the capture probe sequences and universal anchor primers can potentially be used to PCR amplify the captured DNA fragment for cloning and sequence analysis.

In this application, the investigators propose to develop the technology for the identification of DNA fragments from microorganisms that may be involved in metal bioremediation. The specific aims for one year are as follows: (1) Evaluate the bead-based method with respect to sensitivity, sequence discrimination, accuracy and precision in measuring abundances. (2) Determine if the bead-based method can measure abundances of seven genera of bacteria, previously identified to be important for metal reduction. (3) Develop an assay using beads to capture useful genes from environmental samples and prepare the captured material for PCR amplification, cloning and sequencing. (4) Assemble a set of candidate probes for metal reduction genes, and determine if the bead-based method can expedite the development of effective probes for capturing these genes. The sequence length of the capture probe may need to be considered.

Metal and Radionuclide Bioremediation of Mixed Wastes by Starvation Promoter-Driven Combinatorial Bacteria

Metal and Radionuclide Bioremediation of Mixed Wastes by Starvation Promoter-Driven Combinatorial Bacteria

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This grant deals with two issues: purification of bacterial chromate reductases and cloning of their genes, and improvement of starvation promoter expression of *Pseudomonas putida.*

Chromate reductases: Cr(VI) (chromate) is a widespread environmental contaminant. Bacterial chromate reductases can convert soluble and toxic chromate to insoluble and less toxic Cr(III). Bioremediation can therefore be effective in removing chromate from the environment, especially if bacterial propensity for such removal is enhanced by genetic and biochemical engineering. To clone the chromate reductase-encoding gene, we purified to homogeneity (>600-fold purification) and characterized a novel soluble chromate reductase from *Pseudomonas putida*, using ammonium sulfate precipitation (55-70%), anion exchange chromatography (DEAE Sepharose CL-6B), chromatofocusing (Polybuffer exchanger 94), and gel filtration (Superose 12 HR 10/30). The enzyme activity was dependent on NADH or NADPH; the temperature and pH optima for chromate reduction were 80°C and 5, respectively; and the K_m was 374 μM, with a V_{max} of 1.72 μmol/min/mg protein. Sulfate inhibited the enzyme activity non-competitively. The reductase activity remained virtually unaltered after 30 min exposure to 50°C; even exposure to higher temperatures did not immediately inactivate the enzyme. X-ray absorption near-edge structure spectra showed quantitative conversion of chromate to Cr(III) during the enzyme reaction. Physiological studies strongly suggest that the chromate reducatase we have purified has some other role for the bacterium. This premise is supported by the finding that many bacterial enzymes characterized in other contexts have chromate reductase activity — we have cloned one such gene from *Escherichia coli.*

Starvation promoters: The use of these promoters makes it possible to greatly minimize biomass formation and nutrient demand for bioremediation processes. Recognizing the gene that codes for proteins which sense starvation and other stresses in bacteria can greatly assist in manipulating starvation promoter activities in differently stressed DOE sites. We cloned the *flhF* gene of *P. putida,* which encodes a GTP-binding (G-) protein. Its disruption compromises induction of more than 50 starvation proteins and development of the starvation-induced general stress resistance (GR). It appears that this G-protein senses the onset of stresses by changing into its GTP-bound form, thereby activating the starvation/stress promoters. Interestingly, disruption of this gene renders *P. putida* unable to place its flagella at the cell pole and incapable of directional motility; instead it becomes randomly distributed throughout the cell surface. Moreover, overproduction of this gene product greatly increases the number of polar flagella. Thus, this GTP binding protein controls both the stress-resistance response as well as flagellar assembly, opening the way for manipulating motility and chemotactic function as well.

Cellular Response of *Shewanella Putrefaciens* to Soluble and Solid-Phase Metal Electron Acceptors

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The fate and transport of many multivalent metals and radionuclides can be strongly influenced by a phylogenetically diverse group of microorganisms, termed dissimilatory metal-reducing bacteria (DMRB). The DMRB, *Shewanella putrefaciens* MR-1, a facultative anaerobe that displays remarkable respiratory capacity, is amenable to genetic manipulation and is the subject of a DOE-sponsored microbial genome sequencing project. The research proposed herein is intended to develop an understanding, at the genetic level, of how *S. putrefaciens* derives energy by coupling oxidation of organic compounds or H_2 to reduction of either soluble- or solid-phase Fe(III) oxides. In particular, we intend to investigate specialized functions that we hypothesize are required for utilizing solid phase Fe(III) (oxides or oxyhydroxides).

Hypotheses will be tested through the study of differential transcriptional responses associated with the growth and/or respiration of *S. putrefaciens* MR-1 in the presence of soluble- or solid-phase (such as Fe oxides) electron acceptors. Transcriptional activity will be measured by the use of cloned *Shewanella* promoters fused to the GFP reporter gene. Time-course measurements will reveal the sequence of transcriptional events that mediate the response of *S. putrefaciens* to anaerobic respiration via dissimilatory iron respiration. This research will provide important insights into the response *of S. putrefaciens*, at the cellular level, to metals, both soluble- and solid-phase, as electron acceptors. Ultimately, we expect to gain insights into the mechanisms by which this metabolically versatile organism accesses Fe(III) for respiration from insoluble metal oxides.

Probing the Proteome with Capillary Isoelectric Focusing-ESI-FTICR Mass Spectrometry

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Bacterial strains such as *Shewanella putrefaciens* strain MR-1 are key organisms in the bioremediation of metals due to their ability to enzymatically reduce and precipitate a diverse range of heavy metals and radionuclides. Important in these processes is the need to develop improved enzymatic pathways in these organisms. As a first step, the proteome of the organism must be completely characterized. The proteome is defined as the entire protein complement of the cell expressed under a given set of conditions. A single genome can exhibit many different proteomes depending on stage in cell cycle, cell differentiation, response to environmental conditions (nutrients, temperature, stress, etc.), or the manifestation of disease states. While the availability of full genomic reference sequences provides a set of road maps as to what is possible, and measurements of the expressed RNAs tells us what might happen, the proteome is the key that tells us what really happens. Therefore, the study of proteomes under well-defined conditions can provide a better understanding of complex biological processes, which requires faster and more sensitive capabilities for the characterization of cellular constituents.

We are currently developing technologies that will allow the visualization of the protein complement by obtaining comparative displays for the expression of many proteins simultaneously, based upon stable-isotope labeling. Two versions of each protein are generated and analyzed simultaneously, to precisely establish changes in expression. Capillary isoelectric focusing on-line with Fourier transform ion cyclotron resonance mass spectrometry provides a powerful tool to study the changes in expression (i.e., repression or induction) for hundreds of proteins simultaneously. Further characterization of the proteome can be accomplished by characterization of the proteolytic fragments of the proteins in the organism. For many proteins, these proteolytic fragments can be used as unique mass markers for the identification of the proteins in question. Additionally, the sequence of the peptides can be determined as another identification technique. These combined technologies will enable ultra-sensitive proteome-wide expression profiling to evaluate changes in the complete proteome of the iron-reducing bacterium *Shewanella putreficiens* strain MR-1 induced by switching from aerobic to anaerobic respiration with heavy metals and radionuclides.

Optimizing the Metalloregulator MerR for Metallosequestration and Metallosensing

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This is a new project based on our previous work supported by another agency. Its objective is to re-engineer the metal binding domain (MBD) of MerR to recognize metals besides Hg(II), especially those of interest in DOE bioremediation.

Expression of the Tn21 mercury resistance (mer) operon is regulated by a high affinity, high specificity metal-sensing repressor-activator, MerR, which represses transcription of the structural genes, merTPCAD, in the absence of Hg(II) and activates their transcription in the presence of Hg(II). MerR contains three domains: An N-terminal DNA binding domain, a C-terminal Hg(II)-metal-binding domain (MBD), and a coupling domain which lies between them. The MBD consists of a helical region from Cys82 to Cys117 followed by a loop from Cys117 to Cys126. Dimerization of MerR is effected by the formation of a coiled-coil employing these C-terminal helical regions of each monomer. Independent, Hg(II)-binding centers lie at each end of the coiled-coil and are comprised of Cys82 from one monomer and Cys117 and Cys126 from the other. This novel inter-subunit, trigonal metal binding center is highly specific for Hg(II) over other Group 12 metals, Cd(II) and Zn(II). We have previously described 11 single point mutants which allow MerR to respond to Cd(II), but not to Zn(II).

As the next step in manipulating the metal-responsiveness of MerR, we are asking how the loop region between Cys117 and Cys126 influences metal specificity by replacing the loop region of MerR with the ZntR loop region. ZntR, is a MerR-like regulator that is more responsive to Zn(II) and Cd(II) than to Hg(II), but unlike MerR, it contains a histidine residue in the middle of its loop region. Histidines are very often found as ligands to Zn(II) in other proteins, so we hypothesize that the ZntR histidine residue may provide a fourth ligand required for Zn(II)-or Cd(II)-binding. To avoid possible artifacts when using metals other than Hg(II), we have switched from using a hexa-Histidine affinity tag to using a streptavidin affinity tag (Sigma-Genosys) for protein purification. We are screening these mutant proteins for their ability to bind Hg(II), Cd(II), Zn(II), and ions of metals involved in radiation bioremediation, including Co and Pb, using a facile DNA gel mobility shift assay to detect metal-induced allosteric changes in MerR. When mutants exhibiting optimum metal affinity characteristics are identified, their MBD domains will be produced alone (lacking the DNA-binding and coupling domains) and subject to precise metal-binding quantification via equilibrium dialysis and isothermal calorimetry. Further optimization of these minimal MBDs will employ phage display technology.

Genes for Uranium Bioremediation in Desulfovibrio

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Members of the species *Desulfovibrio* have been shown to be capable of uranium reduction. We have initiated a genetic approach to identify components of the reduction pathway to this metal in *Desulfovibrio desulfuricans*. Because experiments from Derek Lovley's laboratory point to the role of hydrogenases and cytochrome c_3 in electron flow to uranium in *D. desulfuricans*, two mutants of the strain G20 lacking a [NiFe] hydrogenase and one lacking the [Fe] hydrogenase have been constructed and screened for uranium reduction. Quantitative assays have indicated that these mutants retained the ability to reduce uranium at the same rate as the parental strain. A mutant of G20 with a plasmid inserted in the *cycA* gene encoding cytochrome c_3 has been generated and characterized. While the growth rate of the mutant on lactate is essentially the same as the parental strain, there is a dramatic decrease in growth on pyruvate. The mutant produces copious hydrogen when incubated with pyruvate. Surprisingly, the *cycA* mutant is still capable of uranium reduction, but does so with a rate that is about 50% that of the wild type, regardless of the electron donor, lactate, pyruvate or hydrogen. We interpret these results to suggest that cytochrome c_3 is involved in the reduction of uranium but that other pathways for reduction are possible. Studies to elucidate the regulation of *cycA* are in progress.

Single-Molecule Studies of Flavin Enzymes

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The high mobility of radionuclides complexed with chelating agents such as ethylenediaminetetraacetate (EDTA), nitrilotriacetate (NTA) and diethylenetriamine pentaacetate (DTPA) represents a major environmental concern at DOE sites. Biodegradation of the chelating agents by microorganisms can help immobilize radionuclides in the environment. Recently, an NTA monooxygenase from an NTA degrading microorganism and an EDTA monooxygenase from an EDTA-degrading microorganism have been purified and characterized. Both NTA monooxygenase and EDTA monooxygenase are $FMNH_2$-utilizing monooxygenase and they need a flavin reductase to supply $FMNH_2$. $FMNH_2$ produced by the reductase is rapidly oxidized by free O_2 to H_2O_2. However, both NTA and EDTA monooxygenases can compete with free oxygen for $FMNH_2$ oxidation. Since $FMNH_2$ is unstable, an immediate question is whether there is metabolic channeling between the reductase and NTA monooxygenase or EDTA monooxygenase. We are studying this question by both conventional biochemistry and single-molecule microscopy.
For biochemistry studies, we need some mutant NTA monooxygenases that cannot oxidize NTA due to their lack of binding to NTA. The mutant proteins will be used to compete with the wild type NTA monooxygenase. If there is metabolic channeling, the flavin reductase and NTA monooxygenase will be in contact and pass $FMNH_2$ directly from the reductase to the monooxygenase. The mutant protein is unable to bind NTA but should still form a contact with the reductase. If there is any metabolic channeling, the mutant protein will quench the wild type monooxygenase for NTA oxidation. We have used a chemical reagent to modify arginine residues. After modification, the enzyme is inactivated. When the enzyme has bound NTA, the enzyme is not inactivated by the chemical reagent. Several arginine residues that are protected by NTA have been identified and mutated to other amino acid residues by site-directed mutagenesis. Eight mutants that cannot oxidize NTA have been obtained. We are in the process of purifying all the mutant proteins to check their ability to quench the wild-type NTA monooxygenase for NTA oxidation. This experiment will provide some evidence on whether $FMNH_2$ is directly channeled from the reductase to the monooxygenase.

We are also using single-molecule microscopy to investigate whether there is metabolic channeling. We have demonstrated that the single-molecule microscopy is a very useful tool to monitor enzymatic reactions associated with the oxidation and reduction of a prosthetic flavin group of enzymes. For NTA oxidation, FMN is not a prosthetic group but a substrate and end-product. We are trying to observe the continuous reduction and oxidation of a single FMN molecule by the reductase and O_2. The emission of the single FMN molecule is monitored as it hops among many reductase molecules and O_2 molecules. NTA monooxygenase will be added to monitor the transfer of $FMNH_2$ from the reductase to NTA monooxygenase. Mutant NTA monooxygenases will also be used in this experiment.

These experiments will provide necessary evidence to prove whether there is metabolic channeling during NTA oxidation. If there is metabolic channeling, we have to use the specific flavin reductase to supply $FMNH_2$ for either NTA monooxygenase or EDTA monooxygenase. If not, any flavin reductase can be used to supply $FMNH_2$ for the two enzymes. This is important information for constructing genetic engineered organisms for the biodegradation of chelating agents.